I. THE EFFECT OF EXCESS OF VITAMINS A, B_1 AND C ON THE ASSAY OF VITAMIN D AND OF EXCESS VITAMIN D ON THE ASSAY OF VITAMIN A

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Several workers have found that the response to a particular dose or to a graded series of doses of one dietary factor is influenced by the amount of another dietary factor given at the same time; e.g. the response to doses of vitamin D when small amounts of mineral phosphates were added to the diet [Bruce & Callow, 1934], the weight response to vitamin D when different amounts of vitamin A were given daily [Coward *et al.* 1932], the weight response to vitamin B_1 when different amounts of autoclaved yeast as a source of vitamins B other than B_1, were included in the basal diet [Coward *et al.* 1933]. Whether these results be interpreted as an "interdependence" of the vitamins or as a "sparing action" of one vitamin on another, it is clear that every factor known to be essential except the one under investigation must be supplied in amounts adequate for all responses whenever an estimation of potency is to be made. Otherwise, in comparisons of a food substance which contains certain quantities of different vitamins with an international standard that contains only one, the vitamins of the food substance may make good the partial deficiencies in the basal diet and thus bring about a bigger response than would be brought about by the one vitamin alone which is being determined. This was also suggested by Bacharach *et al.* [1931], who showed, however, that small additions of carrot, 1 g. daily, to the Steenbock 2965 ration had no influence on the rate of growth of the rats or on the development of rickets with and without doses of vitamin D.

The experiments mentioned above dealt with the influence of partial deficiencies in the basal diet. The influence of an excess of any one vitamin on the determination of another vitamin has been investigated in the experiments described in this paper.

I. *The influence of large doses of vitamins A, B_1 and C respectively on the response of rats to a dose of vitamin D as measured by the line test*

The tests were carried out according to the line test technique on young rats described by Dyer [1931]. All the rats were given a single dose of international standard vitamin D on the first day of test and in addition one half of the rats received a daily supplement of the vitamin under examination for the first 9 days. The animals were killed after 10 days and the line test applied to the distal ends of the ulnae and radii.

The supplements were as follows:

Vitamin A. A solution of carotene (B.D.H.) in coconut oil of such strength that 20 mg. solution contained 20γ carotene was used. The doses of 40γ carotene per rat per day were administered by drops directly into the animal's mouth from a calibrated dropping tube at 40–45° (Table I, exp. 1–4).

Vitamin B_1. A vitamin B_1 adsorption product on acid clay, containing 100 I.U. vitamin B_1 per g. was used. The doses of 10 or 15 I.U. were weighed daily in separate portions and moistened with a little water to prevent scattering of the powder. No instance of an animal refusing to eat its dose occurred (Table I, exp. 5).

Vitamin C. A solution of ascorbic acid in distilled water was prepared daily immediately before use and doses of 0·15 mg. were delivered directly into the animal's mouth from a micrometer syringe (Table I, exps. 6 and 7). This test was included in spite of the generally accepted fact that the rat does not need a supply of vitamin C in the diet.

The results are given in Table I.

Table I. *The effects of vitamins A, B_1 and C respectively on the response of rats to a dose of vitamin D*

1	2	3	4	5		6
				Average healing of the group of rats		
Exp.	Daily dose of supplementary vitamin given to one member of each pair of rats for 9 days	Dose of vitamin D given to all rats on the 1st day of test I.U.	No. of pairs of rats	Vitamin D + supplement	Vitamin D alone	Apparent dose ratio $\frac{\text{vitamin D + supplement}}{\text{vitamin D alone}}$
1	40γ carotene	10	11	1·64	2·05	0·74
2	,,	5	15	2·07	1·93	1·10
3	,,	5	15	2·07	1·80	1·21
4	,,	5	16	1·60	1·66	0·95
5	10 or 15 I.U. vit. B_1 100 units in 9 days	10	12	2·68	2·50	1·14
6	0·15 mg. ascorbic acid	10	12	2·59	2·17	1·34
7	,,	5	20	2·50	2·28	1·17

In col. 5 of Table I is given the average healing brought about by the dose of vitamin D plus the supplement and that brought about by the dose of vitamin D alone in each experiment. Since the relation between the amount of healing and dose of vitamin D given is not a straight line but a logarithmic curve, each ratio for healing can, by means of this curve, be converted into the apparent dose ratio. This ratio is given in col. 6.

In view of the known variation in all biological responses and the relatively small numbers of animals used, none of these ratios can be considered significantly different from unity. It must be concluded that very large doses of vitamins A, B_1 and C respectively have no influence on the amount of healing brought about by a small dose of vitamin D. It is therefore unnecessary, in determinations of the vitamin D content of a substance, to take into consideration the amounts of these vitamins likely to be present in that substance.

II. *The influence of a large dose of vitamin D on the weight response of rats to a dose of vitamin A*

The technique used in the vitamin A test has been fully described by Coward *et al.* [1930]. Young rats of about 30 g. weight and 18–21 days old were given the vitamin A-free diet immediately after weaning. The sexes were segregated and groups of about 7 rats of the same sex were kept together in a cage. The young rats were weighed at first weekly and after the first 4 weeks twice weekly, until

they ceased to grow and showed a loss in weight. As the animals became ready for dosing each rat was put into a separate cage. Three groups were formed. The litters were divided as equally as possible among the three groups but precedence was given to the date of depletion rather than to the genetic constitution so that all the rats ready on any one day were divided equally among the three groups.

During the depletion period, vitamin D was supplied as a solution of irradiated ergosterol in olive oil of such strength that 8 I.U. vitamin D were contained in 20 mg. solution. A single drop of this solution was fed to each rat once a week. This had been shown to be an ample allowance for rats receiving the vitamin A-free diet [Coward *et al.* 1932].

When the rats became steady in weight they were given doses of vitamin A twice a week (three doses on one day and four doses 3 days later [Coward & Key, 1934]. In addition they were given a single dose of vitamin D per week, as during the depletion period, containing the following amounts: Group 1, 8; Group 2, 25; Group 3, 100 I.U.

The vitamin D used during the test period was a solution of calciferol in olive oil of such strength that the required weekly dose was contained in 20 mg. solution.

The dose of vitamin A chosen was 2 mg. daily of a particular sample of cod liver oil, known in this laboratory as "oil Z". The dose was small so that either a depressing or an enhancing effect of the excessive doses of vitamin D could be detected. The oil contains approximately 1500 I.U. vitamin A and 100 I.U. vitamin D per g. The rats were therefore receiving a daily dose of 3 I.U. vitamin A. At the end of the first week the dose was reduced to 1 mg. cod liver oil, i.e. to 1·5 I.U. vitamin A per day. The rats would also receive from the cod liver oil 1·4 I.U. vitamin D during the first week and 0·7 I.U. vitamin D per week for the remaining 4 weeks of test. The animals were weighed once a week during the test period. Table II shows the average increases in weight of the three groups of rats.

Table II. *Growth response to a dose of vitamin A with variations in the vitamin D supplied*

Dose of vitamin D in the basal ration. I.U. per week	No. of rats ♂	No. of rats ♀	Total increase in wt. (g.) 1st week 2 mg. c.l.o. oil Z daily	Total increase in wt. (g.) 2nd–5th weeks 1 mg. c.l.o. oil Z daily	Total increase in weight during 5 weeks g.	Mean increase in weight per rat per week g.
8	4		8	68	76	3·8
		10	39	142	181	3·6
25	6		12	96	108	3·6
		11	26	170	196	3·6
100	4		20	60	80	4·0
		10	33	191	224	4·5

There is evidently no difference in response with the three levels of vitamin D intake and it may be concluded that giving a large excess of vitamin D, even twelve times the amount which had been considered ample, had no influence on the weight response of a group of rats to a dose of vitamin A during a 5 weeks' feeding period.

Although previous workers have shown that the effect of a small dose of a vitamin may be diminished by a partial deficiency of another vitamin in the diet

given during the test, the effect of the small dose cannot be enhanced indefinitely by giving more and more of the other vitamins. Thus there is no "sparing action" in the influence of one vitamin on another. Further, excessive amounts of one vitamin in the diet do not diminish the response to a small dose of another vitamin. There is, therefore no danger of the determination of one vitamin in a substance being spoilt by the presence of even excessive amounts of other vitamins.

SUMMARY

1. The response to a small dose of vitamin D (as measured by the line test) was not influenced by giving at the same time excessive doses of vitamins A, B_1 or C.

2. The weight response to a small dose of vitamin A was not influenced by giving a graded series of excessive doses of vitamin D.

3. In assaying vitamin A or vitamin D, it is unnecessary to consider the possible presence of another vitamin in the substance under test, provided that the basal diet is adequate.

The writers wish to thank Dr Katharine Coward and Prof. Burn for helpful advice and criticism during the progress of this work.

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II. CONDITIONS INFLUENCING THE STORAGE OF VITAMIN A IN THE LIVER

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It is generally recognized that in most animals the liver is the chief depot of vitamin A reserves. It has long been known that these reserves are utilized when the diet of the animal contains insufficient vitamin, but an important fact was revealed when Davies & Moore [1935] showed that the conside able accumulation of vitamin which occurs in the liver when the diet contains a large excess is rapidly dispersed when an A-deficient diet is given. The rate of disappearance of this reserve was far greater than that which might reasonably represent the daily requirements of the animal.

The fate of this metabolized vitamin is unknown, although preliminary studies carried out in this laboratory [Santos Ruis, 1935] suggested that a part is excreted in the urine as an unsaturated breakdown product. Clearly the observations of Davies & Moore have an important bearing not only on the question of the cause of the variations in the vitamin A content of liver oils used for therapeutic purposes but also on the fundamental problem of the function of the reserves of vitamin A in the liver.

A large amount of work on the variations in the vitamin A potency of fish liver oil has shown that influx of fat into the liver or its subsequent mobilization will change the concentrations of vitamin, but it is not known whether this is merely a dilution effect or whether the vitamin may be directly concerned, more particularly when fat leaves the liver. The investigations of Drummond [1919], confirmed by Emerique [1932], indicated that vitamin A is not obviously concerned with the mechanisms for absorption and utilization of fat in the organism, whilst recently Green [1934] has recorded that a diet very rich in fat does not accelerate the utilization of the vitamin and that the more obvious aspects of fat metabolism appear to be unrelated directly to vitamin A.

A new experimental method of great value in investigating the function of the liver as a storage organ for fat was provided when it was discovered that choline would prevent the accumulation of fat in the liver of rats living on unbalanced diets rich in fat or cause the excess to disperse if the livers had already become fatty [Best, Huntsman & Solandt, 1932; Best & Ridout, 1933]. The action of choline is primarily on the glyceride fraction, the increased deposition of cholesterol which occurs in animals fed on fat-rich diets, or more strikingly, after administration of unusually large amounts of the sterol itself, being less affected [Best, Channon & Ridout, 1934; Channon & Wilkinson, 1935; Best & Ridout, 1935].

It was thought that a series of experiments in which fatty livers were produced in animals on high or low intakes of vitamin A and in which the fat so deposited could afterwards be dispersed by the "lipotropic" action of choline might throw some light on the influence which these changes had on the amount of vitamin A stored or utilized.

Experimental

Litters of rats, seven in each, from 3 weeks to a month old were used. They were fed on a balanced stock diet until they weighed from 100 to 120 g. They were then distributed, on a litter basis, between seven groups and given one of the following diets. The protein content of the diets was kept low in order to diminish the influence of the "lipotropic" effect which proteins or associated substances exert [Beeston, Channon & Wilkinson, 1935]. The casein was extracted with alcohol and ether.

Diet*	Casein	Starch	Glucose	Lard	Cholesterol	Choline hydrochloride
Control normal (N.)	10	18	50	12	—	—
High fat (H.F.)	10	10	30	40	—	—
High fat with choline (H.F.C.)	10	10	30	40	—	2
High cholesterol (H.C.)	10	10	40	28	2	—
High cholesterol with choline (H.C.C.)	10	10	40	28	2	2
Low fat (L.F.)	10	25	55	—	—	—
Low fat with choline (L.F.C.)	10	25	55	—	—	2

* We wish to acknowledge gratefully much helpful advice given by Prof. H. J. Channon with regard to the selection of appropriate diets and on other matters.

Diet for all groups contained:

Yeast extract	...	5
Salt mixture	...	5

In addition each animal received ~~25 mg.~~ of a standardized cod-liver oil every week. It contained 1800 I.U. vitamin A per g. The cholesterol was dissolved in the lard used for inclusion in diets H.C. and H.C.C.

The animals were fed on these diets for 4 weeks. Growth and food consumption were recorded.

Diet	Animals ♂	Animals ♀	Av. daily food intake g.	Av. increase g.
N.	4	6	11·8	17·0
H.F.	5	7	11·4	11·7
H.F.C.	5	6	7·7	15·7
H.C.	4	6	9·6	12·3
H.C.C.	3	8	7·8	17·7
L.F.	7	5	11·2	14·9
L.F.C.	4	7	11·0	13·4

At the end of the period the animals were starved overnight and then killed by a blow on the head. The livers were drained and weighed individually. Those belonging to rats of the same diet group were pooled, thoroughly minced and a small sample taken for moisture determination. The rest was divided into two weighed portions, each of which was ground with anhydrous sodium sulphate and the dry powder immediately extracted with chloroform in a Soxhlet apparatus.

The extract was evaporated under reduced pressure. This gave the "fat" content of the liver. The "fat" of one extract was used for estimation of total phosphorus by Tisdall's method as modified by Peters & Van Slyke [1931–2]. The "fat" from the other sample was used for estimation of the unsaponifiable fraction. One-half of the unsaponifiable matter so obtained was used for estimating cholesterol by precipitation with digitonin, whilst the other half was examined for vitamin A by means of the Adam Hilger "Vitameter A". It was originally intended to estimate the vitamin A in the whole oil as well as in the unsaponifiable matter but it was soon realized that the results thus obtained

were useless, owing to the large amount of irrelevant absorption. The "Vitameter A" was standardized against fish liver oils previously examined spectroscopically for absorption at 3280 Å.

The results of the analyses are given below.

Composition of rat liver oils and rat livers

Diet	N.	H.F.	H.F.C.	H.C.	H.C.C.	L.F.	L.F.C.
Av. wt. of livers, g.	5·60	7·05	4·97	11·71	5·74	5·47	4·20
Fat content % of livers	8·95	26·21	5·52	41·28	18·13	5·31	4·68
Water content % of livers	67·20	54·80	69·20	44·00	60·70	69·70	69·25
Lecithin g. per 100 g. liver	3·00	2·73	3·10	1·88	2·99	2·97	2·55
Unsaponifiable % of oil	7·81	5·63	8·90	18·64	40·39	9·26	11·97
Sterol % of oil	4·71	3·30	4·78	16·9	33·3	4·82	6·00
$E^{1\%}_{1\,cm.}$ 328 mμ	0·18	0·11	0·39	0·048	0·14	0·34	0·40

Most of the figures are in agreement with the observations of Best & Channon and their associated workers; for example, the lecithin percentages confirm the observations of Best, Channon & Ridout [1934] that the proportion of phosphatide falls with fatty infiltration.

The vitamin A potency of the liver oils is, generally speaking, inversely proportional to the amount of fat present. These figures may, however, merely be an expression of dilution and concentration of the vitamin. They do not tell us what total amount was present. This may be estimated from the product $E^{1\%}_{1\,cm.} \times$ % fat × wt. of liver (E.F.L.), which for the seven groups was

	N.	H.F.	H.F.C.	H.C.	H.C.C.	L.F.	L.F.C.
E.F.L.	9·64	20·4	10·7	23·3	14·5	9·85	7·87

This calculation does not take into account the size of the animals, but since they were all of the same age, had received the same quantity of vitamin A during the 4 weeks of experimental feeding and had been kept under identical conditions, this is regarded as unimportant. The figures reveal that the storage of fat or of cholesterol in the liver tends to increase the total amount of vitamin. This cannot be merely a question of better absorption when much fatty material is present in the diet, because the animals receiving no other fat than the 3·6 mg. cod liver oil daily (L.F. and L.F.C.) stored as much vitamin as did the control group (N.); moreover, there is no reason to think that the cod liver oil was not fully absorbed in all cases.

Influence of fat mobilization

A second series of experiments was then carried out to ascertain what happens to vitamin A already present in the liver when associated fat is caused to leave under the "lipotropic" influence of choline.

The main comparison was between basal diets containing much (H.F.) and little (L.F.) fat. Instead of giving cod liver oil as a source of vitamin A, a concentrate was employed, so as to enable a larger dose to be given. The amount given to the rats of the appropriate groups was 2 mg. daily. Spectroscopic measurements showed that this corresponded to an intake of approximately 3500 I.U. a day. It was dissolved in 0·025 g. of coconut oil and administered directly to the animals.

The basal "high-fat" diet (H.F.) was of the same composition as that given on p. 6; the composition of the "low-fat" diet was, however, modified so that it included 30% of casein and 50% of starch. It did not contain glucose.

Seven groups of animals were studied. They were treated as follows:

Group 1 was fed for 4 weeks on the "low-fat" (L.F.) diet. This was regarded as a control group.

Group 2 received the L.F. diet supplemented with vitamin A for 4 weeks after which it was divided into three subgroups.

One, *Group 3*, was transferred to the basal L.F. diet for 9 days.

Another, *Group 4*, was given the low-fat-choline (L.F.C.) diet for the same period, whilst the third was killed.

Group 5 received the "high-fat" (H.F.) diet supplemented with vitamin A for 4 weeks and was then similarly divided into three subgroups.

One, *Group 6*, was given the basal H.F. diet.

The second, *Group 7*, was transferred to the "low-fat" diet supplemented with choline (L.F.C.), whilst the third was killed. In all cases the period was 9 days.

Group	Original diet 4 weeks	Subsequent diet for 9 days	No. of animals	Av. wt. of rats g.	% fat in livers	% Unsap. in liver fat	$E^{1\%}_{1\,cm.}$ 328 mμ in liver fat	E.F.L.
1	L.F.	—	5	143	4·98	9·00	0·49	13
2	L.F. + Vit. A	—	5	145	5·13	9·55	12·96	400
3	L.F. + Vit. A	L.F.	6	139	5·17	8·20	1·03	26
4	L.F. + Vit. A	L.F. + chlorine	4	185	4·76	9·09	1·42	48
5	H.F. + Vit. A	—	6	139	11·12	9·57	14·66	900
6	H.F. + Vit. A	H.F.	6	125	8·71	7·96	7·83	300
7	H.F. + Vit. A	L.F. + chlorine	6	150	6·28	11·26	2·79	120

E.F.L. represents, as before, the product of the $E^{1\%}_{1\,cm.}$ (328 mμ) × % fat × wt. of liver, i.e. it is an expression of the total amount of vitamin stored. The first point to observe is that the percentage of fat in the liver was not so high in the animals fed on the H.F. diet as it had been in the previous experiment. Such variations are not uncommon.

The results again show that storage of vitamin A in the liver is facilitated by the presence of fat in the diet and its subsequent deposition in the liver. Considerable storage of the vitamin occurs as a consequence of giving a large excess in the diet, but such reserves are rapidly depleted, as Davies & Moore demonstrated, when the vitamin is no longer administered. In as short a period as 9 days the reserves accumulated on a diet rich in vitamin A but poor in fat are almost completely dispersed. This loss occurs without there being any material change in the fat content of the liver. A similar and striking reduction in the amount of vitamin A stored in the liver occurs in animals fed on a diet rich both in the vitamin and fat when the vitamin is withheld. The depletion appears to be accelerated, however, by causing a partial dispersal of fat in the liver by the administration of choline. This indicates that fat leaving the liver may take with it vitamin A. The matter requires further study but it is interesting to note that Dann [1934] has recorded that female rats fed on a fat-rich diet (20%) appear to pass on more vitamin A to the foetuses during gestation than those fed on a diet containing only 1%.

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III. CURATIVE ACTION OF NICOTINIC ACID ON PIGS SUFFERING FROM THE EFFECTS OF A DIET CONSISTING LARGELY OF MAIZE

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In the September number of the *Journal of the American Chemical Society* the announcement was made by Elvehjem *et al.* [1937], that nicotinic acid cured dogs of "black tongue" induced by feeding upon a Goldberger "pellagra producing" diet of which maize was the chief ingredient [Goldberger *et al.* 1930]. The composition of the diet was the same as that of the diet used by Koehn & Elvehjem [1937]. Their experiments indicate that nicotinic acid or its amide or some combination of the latter, is the active constituent of Goldberger's "pellagra-preventive". The idea that another component of the vitamin B_2 complex in addition to lactoflavin is intimately concerned with one of the types of cellular respiration is an alluring one.

Continuing the work of Birch, Chick & Martin [1937] of the previous year, we had been attempting to discover the active principle in an autoclaved yeast extract which rendered a Goldberger maize diet satisfactory for the rearing of young pigs and had found that this principle possessed chemical and physical properties similar to those originally described by Goldberger as appertaining to his "pellagra-preventive", notably the property of being adsorbed on fuller's earth [Goldberger *et al.* 1928].

We had at hand two animals (Nos. 18 and 26) which had become very ill as a result of feeding upon the unsupplemented diet, which consisted of whole ground white maize 77·5, peameal 10·5, purified casein 6·5, cod liver oil 3 and salt mixture 2·5 parts. Various fractions prepared from yeast extract had been given without effecting any material improvement and, by the time we tried nicotinic acid, their weights were the same as they had been 11 and 7 weeks previously and were falling rapidly (see Fig. 1). Both pigs had severe diarrhoea and refused food. Their skins were a dirty yellow colour instead of the rosy pink of healthy young pigs and were covered with scabs of heaped epithelial cells matted together by inspissated serum. In No. 18 the dermatitis was more severe; she had lost most of her hair and what remained could be easily detached. In addition she had paresis and spasticity of the muscles of the hind quarters indicating lesions in the spinal cord. From previous experience of animals in the condition of Nos. 18 and 26 we anticipated that they would die within 2 or 3 days.

As both animals had ceased to eat, a solution of nicotinic acid which had been brought to *p*H 7·4 with NaOH was injected intramuscularly. No. 18 had two, and No. 26 three, injections containing 100 mg. of nicotinic acid, at intervals of 3 days. Subsequently 60 mg. was given to each daily with the food.

The effect was striking. The appetite returned within 24 hr. of the first injection, the diarrhoea abated and they began to increase in weight (see Fig. 1). The scabs on the skin began to be detached after 1 week, leaving clean, healthy skin. At the same time the colour of the animals improved gradually and after

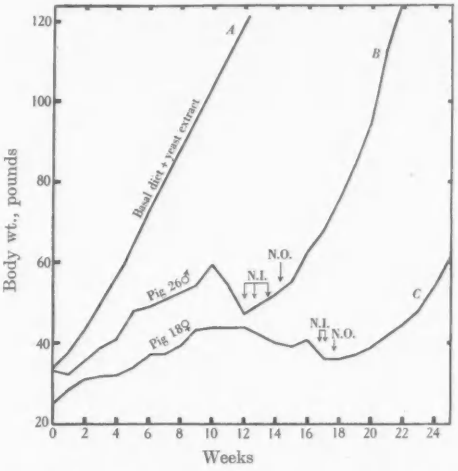

Fig. 1. Curve A. Average weight curve of pigs on the basal maize diet + yeast extract corresponding to 25 g. dry yeast, daily. Curves B and C. Weight curves of pigs 26 and 18, respectively, on the unsupplemented basal diet; at N.I., 100 mg. nicotinic acid injected; from N.O. 60 mg. nicotinic acid given daily by mouth.

6 weeks of treatment was that of healthy pigs. What remained of the hair of No. 18 was gradually shed and a fresh growth of hair was observed 3 weeks after the commencement of the treatment. After 6 weeks No. 18 still showed some awkwardness of movement, but the spasticity had disappeared and she was active and agile.

The amount of nicotinic acid injected was not too much for the desperate condition of our depleted animals but the doses subsequently administered in the food may have been more than was required.

We are at present occupied with experiments to ascertain the amount which it is necessary to add to the basal diet to rear healthy pigs. The results of these experiments will not be forthcoming for some months, so it seemed desirable to record our confirmation of the experiments of Elvehjem and his colleagues on dogs, by these observations on pigs, especially as one of us [see Macrae & Edgar, 1937] had found, in experiments with rats, that neither nicotinic acid, nicotinic amide nor co-dehydrase II could replace either the filtrate from an autoclaved extract of yeast after treatment with fuller's earth, or the eluate from the earth.

Whether nicotinic acid is a necessary constituent of the rat's diet was not ascertained by the experiments of Macrae & Edgar but, as rats thrive on the maize diet we employed, whereas pigs die, a quantitative difference in the nicotinic acid required by the respective animals is at least indicated.

DESCRIPTION OF PLATE I

The upper photograph shows the appearance of pig no. 18 when six months old after 17 weeks on the maize+casein diet. Her weight was then only 36 lb.

The lower photograph shows the same pig three months later, weight 129 lb. The diet had been the same in the interval except that 60 mg. of nicotinic acid had been added to her daily ration.

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NOTE

Since this paper was written, Fonto, Helmer, Lepkovsky and Jukes have reported (*Proc. Soc. exp. Biol. & Med.* Nov. 1937, **37**, 405) the treatment of four pellagrins with 0·5 and 1·0 g. nicotinic acid daily. The patients were in hospital and their diet was one on which pellagrins do not improve. All were cured. The response to the administration of nicotinic acid was striking and immediate and resembled in some details what we observed in pigs.

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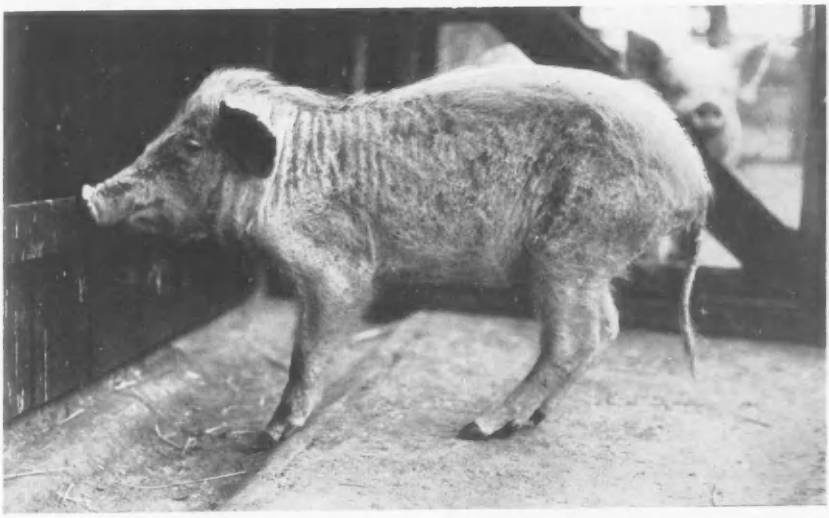

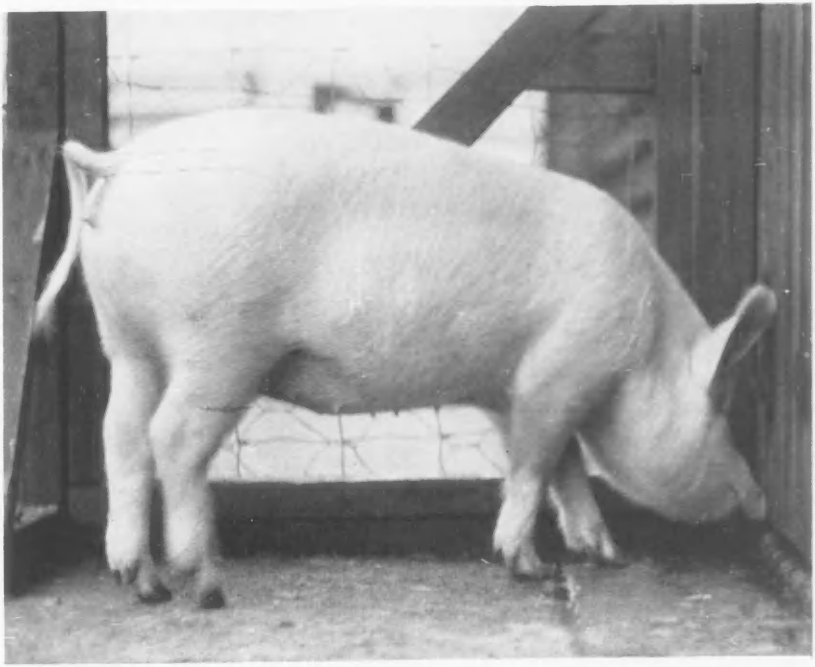

UNIV. OF MICH.

IV. A WET ASHING METHOD FOR THE ESTIMATION OF SODIUM IN FAECES

By DAVID STIVEN

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(*Received 21 November 1937*)

In the course of investigations in this laboratory on the excretion of Na in tuberculous patients, there arose the problem of dealing with many specimens of faeces. The dry ashing method recommended by Butler & Tuthill [1931] has its disadvantages and to avoid these the following method was devised; it has been found to be relatively rapid and to give accurate results. The essential differences in procedure are that wet ashing is employed and that baryta and alcohol are added to the faecal suspension to remove soluble phosphate and organic matter. Since all the Na (and K) salts are soluble in dilute alcohol, the use of a fraction of the alcoholic filtrate from the faecal suspension is considered to be justified, on the assumption of a uniform distribution of the Na between the precipitated faeces and the supernatant fluid.

Procedure

The faeces are transferred from the containers to a 2 l. conical flask fitted with a rubber stopper through which pass an ordinary funnel with a cotton wool plug to act as a dust filter and a bent tube connected to an aspirator. The water escaping from the aspirator is led by tubing down to the bottom of the trap of the sink. During the whole process of heating the faeces a current of air is sucked through the flask to prevent the escape of any odour into the room. In transferring the faeces to the flask and washing out the containers, not more than 300 ml. water should be used. It is a disadvantage to use any more water than is just required to give a suspension that will not "catch" on the bottom of the flask during boiling. If too much water is used, the concentration of alcohol in the diluted faecal suspension will be too low to precipitate the organic matter and the result will be slow filtration and a turbid filtrate. The mixture of faeces and water is now brought to the boil, the flask being rotated at intervals to aid the maceration of the faeces. Only gentle boiling must be allowed on account of frothing. A few minutes' boiling usually suffices to give a uniform suspension and at this stage about 5 g. $Ba(OH)_2,8H_2O$ dissolved in hot water are poured into the mass and the whole is well shaken. After a few minutes' boiling the reaction to phenolphthalein is tested and if it is not alkaline more baryta should be added. The flask is now cooled and the suspension transferred quantitatively to a 1 l. flask; the 2 l. flask is rinsed out and the volume is made up to the mark with rectified spirit. If the stool is small and maceration in about 150 ml. water has been possible, it can be made up to 500 ml. with alcohol. This alcoholic suspension is allowed to stand overnight and after adjusting the volume to the mark it is filtered through an ordinary filter paper. About 800 ml. of filtrate can as a rule be obtained, but centrifuging gives a larger volume.

So low is the Na content of faeces as a rule, that it will be found necessary to use as much of this filtrate as can be obtained. The whole stool may contain

only 15 mg. Na. Since 2–3 mg. Na are a convenient amount to use for precipitation as the triple salt from 20 ml. of the solution of uranyl zinc acetate, and this amount of Na must be contained in not more than 2 ml., a considerable volume of the alcoholic filtrate is necessary for each estimation. The method that has been found most practicable is to ash the residue from 700 to 800 ml. of the filtrate, make this up to about 15–20 ml. and use 2 ml. of this for precipitation of the Na. If the Na content is too high, 1 ml. can be used with 20 ml. of the solution of the double salt.

To carry out the ashing, transfer 700–800 ml. of the alcoholic filtrate back to the 2 l. flask and boil off the alcohol with the air current running. Remove all the alcohol and reduce the volume to about 100 ml. At this stage, with the solution still boiling, add drop by drop 10 ml. 10*N* H_2SO_4 to precipitate the excess barium as sulphate. If frothing should start it can be checked by adding a few drops of conc. HNO_3. When the solution begins to blacken, it is transferred quantitatively to a 300 ml. Kjeldahl flask and the wet ashing is completed in the usual manner by well-timed additions of conc. HNO_3. To the pale yellow solution are now added about 5 ml. water and the mixture is again gently boiled to expel nitrous fumes and to aid the reprecipitation of the barium sulphate that tends to redissolve in the concentrated acid. The contents are now transferred by pipette to a stoppered graduated 25 ml. cylinder. The Kjeldahl flask is rinsed out with 2 ml. lots of water which are transferred each time to the stoppered cylinder by the same pipette. This rinsing is continued until 15–20 ml. in all have been collected in the cylinder.

The volume is noted, the contents are shaken up thoroughly and the solution is filtered through a fine paper to remove the barium sulphate. The Na in 2 ml. lots of this filtrate is estimated by the method described by Butler & Tuthill [1931] for urines of ordinary Na content.

The Na in the sample of faeces used can be calculated thus:

$$\text{Total Na in stool} = \frac{\text{Wt. triple salt}}{67} \times \frac{\text{Vol. to which ashed material is made up}}{2} \times \frac{1000}{\text{Vol. of alcoholic filtrate used}}.$$

An estimation carried out on the reagents used in this laboratory in the quantities described above showed that about 3 mg. Na were added from this source. This amount must be deducted from the result to get the true Na content of the faeces.

The recovery of added Na was investigated by taking a 2 days' stool, macerating it and measuring out two equal volumes of the suspension. To one was added an accurately weighed amount of pure fused NaCl. Both samples were treated as described above. Of 48·4 mg. Na added in one experiment, 47·6 were accounted for in the estimation; in a second experiment the recovery was 54·2 mg. from a sample to which had been added 53·5 mg.

The daily excretion of Na in the faeces of normal individuals has been found to range from 16 to 75 mg.

SUMMARY

The Na content of faeces has been estimated by removal of free phosphate as barium phosphate, precipitation of the organic matter by alcohol and wet ashing of the alcoholic filtrate.

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V. STUDIES IN THE CHOLESTEROL CONTENT OF NORMAL HUMAN PLASMA

VII. AN IMPROVED MACROMETHOD FOR THE ESTIMATION OF CHOLESTEROL BY DIGITONIN

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The methods in use for the estimation of cholesterol and its esters in the tissues are liable to many quite distinct sources of error. These errors may arise through faulty sampling, unsuitable methods of preparing the tissues for extraction, inadequate extraction owing to the adsorption of sterols on the proteins [Gardner & Gainsborough, 1927], incomplete hydrolysis of the esters [Gardner & Fox, 1924], incomplete extraction of the alkaline fluids after hydrolysis and lastly through defects inherent in the methods of estimation of the cholesterol in the extracts, for example, the slight but definite solubility of cholesterol digitonide in the solvents used.

Sperry & Schoenheimer [1935] claimed that oxalated plasma contained considerably smaller amounts of both total and free cholesterol per 100 ml. than either heparinized plasma or serum from the same sample of blood. The average difference between the amounts of total cholesterol in the oxalated and heparinized plasmas was 15·3%. We were much surprised at these results and made a series of duplicate analyses of blood plasma by our usual method [Gardner & Gainsborough, 1927], to determine the magnitude of the probable experimental error, and also to test the above findings of Sperry and Schoenheimer. Our investigations led to some modifications of the method we had previously described, and in this paper we give an account of these experiments and the reasons for the change.

Though many duplicate results obtained by our original method were satisfactory, there were cases in which estimations of the ester showed divergences. We think that these were due to cholesterol being washed away in the soapy alkaline solution obtained after saponification, in which it is known to be slightly soluble. Some illustrative results are shown in Table I.

Table I. *Duplicate analyses of plasma cholesterol.*

Sample	Plasma cholesterol, mg. per 100 g. (old method) Free	Ester	Total	% total as ester
A. A. 1	57	164	221	74·2
	59	173	232	74·6
2	49	152	201	75·6
	49	134	183	73·2
3	51	136	187	72·7
	49	150	199	75·5

It will be noted that whereas the duplicate estimations of the free cholesterol agree to within 4%, the duplicate estimations of the ester cholesterol differ by as much as 10%, and that the latter difference is masked in the calculation of the percentage of the total as ester.

The saponification mixture is now rendered acid, and we extract the fatty acids and cholesterol immediately; it is important that no delay occur at this stage in order to avoid the re-esterification of the cholesterol which will take place in the presence of strong acid. We now generally obtain higher values for the ester fraction and a more constant relation between the free and ester fractions in normal subjects, as found by Sperry [1936].

This constant relation is, however, by no means a general rule; occasional cases are encountered in which the free cholesterol content of the plasma is extremely low. Such results are probably not due to error in the analyses as they have been confirmed in many cases by duplicate estimations. They have occurred in pathological conditions which, however, throw no light on their origin. The following might be quoted as examples:

Table II

Case	Disease	Plasma cholesterol, mg. per 100 g. Free	Ester	Total	% total as ester	Method
E. C.	Gastric ulcer	6	155	161	96·3	New
		5	152	157	96·8	New
E. F.	Hyperpiesia	3	129	132	97·6	Old
		6	121	127	95·7	Old
A. P.	Carcinomatosis	6	185	191	96·9	New
		7	186	193	96·4	New

In one case an effort was made to trace the cause by observing the effect of dietetic variations, with the following uninformative results:

Table III. *Effect of dietetic variations on the plasma cholesterol of case B.C.*

Period days	Diet	Plasma cholesterol, mg. per 100 g. (old method) Free	Ester	Total	% total as ester
	Normal hospital diet	8	125	133	94·0
		10	144	154	93·5
3	Very high carbohydrate, low fat	45	124	169	73·4
		48	123	171	71·9
5	Very high fat, low carbohydrate	45	160	205	78·0
		43	145	188	77·1
7	Normal hospital diet	26	156	182	85·7
		23	151	174	86·8
16	Normal hospital diet	30	98	128	76·6
		28	88	116	75·9

We note that Sperry [1937] found some marked deviations from normal, but apparently did not trust his findings (three analyses) and omitted them from his tables.

A further source of error was noted in the old procedure, for when the original alcohol-ether extract of the plasma was evaporated to dryness, the fatty residue was not stable, and further esterification of the free cholesterol occurred slowly on standing. In one case in which the residue stood at room temperature

it was found that 10 % of the free sterol was esterified in the first three days, and 28 % at the end of a week. We now take the obvious precaution of not allowing the extract to stand.

Furthermore, plasma for the estimation of cholesterol should not be allowed to stand for any period before analysis, as there may be an increase of the ester fraction at the expense of the free. Sperry [1935] finds a considerable degree of esterification to occur in incubation experiments at 37°, and though we can confirm this change, we found that only about 3 mg. of sterol per 100 g. of plasma are so changed, compared with the very much higher figures quoted by Sperry. We could detect this change in heparinized plasma and in serum, but not in oxalated plasma.

In order to avoid the above errors, we now use the following method, which has been in use in this laboratory with satisfactory results for nearly two years.

Method

5–10 g. of serum or plasma are run in a thin stream into a mixture of 180 ml. absolute alcohol and 60 ml. ether contained in a 500 ml. Erlenmeyer flask which is rotated briskly; the fluid is brought to the boil and after cooling is allowed to stand overnight. The supernatant liquid is filtered through an 11 cm. No. 41 Whatman filter paper and the proteins are transferred to the paper, pressed dry and returned to the flask; this protein is then extracted successively with about 50 ml. portions of boiling alcohol-ether mixture, ether, boiling alcohol and finally ether, and the combined filtrates are evaporated to dryness; the residue is immediately extracted with three successive portions of about 25 ml. absolute alcohol, the fluid being filtered through a small filter into a 150 ml. beaker. A solution of digitonin in absolute alcohol is then added in amount approximately 10 % in excess of the probable amount required; the solution is heated to boiling point and after cooling to room temperature water is added until a turbidity appears, as recommended by Fex [1920]. After standing overnight, the fluid is evaporated to dryness in the water bath and the nearly dry residue is extracted three times with 20 ml. portions of dry ether, each portion being filtered through a weighed Gooch crucible. These ether washings, combined with the ether washings of the small filter paper used above, are returned to the original extraction flask for the subsequent estimation of the ester. The residue remaining in the beaker is transferred to the crucible with boiling water and is washed with boiling water until the filtrate no longer froths on shaking. The crucible is dried in a water oven overnight and then in an air oven at 120° to constant weight; it is weighed in a closed weighing bottle (with another similar one on the opposite pan), as cholesterol digitonide is hygroscopic. The total ether washings are made up to about 100 ml., a considerable excess of sodium ethoxide in especially pure alcohol is added and the mixture is refluxed at about 40° on an electric heater overnight. Water is then added, the liquid is made acid to Congo red with strong HCl and saturated with NaCl in order to effect a good separation in the subsequent extraction. The fluid is then extracted with ether in a separating funnel, the acid liquor being transferred to a second funnel where two further extractions are made with ether, which is added to that in the first funnel. The acid layer is extracted once more with ether and is discarded. The main ether washings in the first funnel are shaken out with distilled water until free from chlorides and the aqueous layer is re-extracted with the ether in the second funnel. The combined ether extracts are evaporated to dryness, the residue is taken up in three successive portions of absolute alcohol and the cholesterol is estimated as above.

The digitonin used was the British Drug Houses product which is completely soluble in water; new specimens of digitonin, particularly those of unknown origin, should be tested for solubility and estimated against pure cholesterol.

The Gooch crucibles were packed with a layer about 3 mm. thick of powminco asbestos, which was well macerated with alcohol until all the fibres were broken down to a fine paste; the pad was washed with a jet of alcohol to remove all loose fibres and the crucible was dried in the air oven at 120°.

Using the above method, we obtained close agreement between duplicates, as indicated by some typical results in Table IV.

Table IV. *Duplicate analyses of plasma cholesterol.*

Sample	Plasma cholesterol, mg. per 100 g. (new method)			% total as ester
	Free	Ester	Total	
1. M. J.	54	164	218	75·2
	53	159	212	75·0
2. L. H.	66	217	283	76·7
	65	221	286	77·3
3. R. M.	55	142	197	72·1
	53	144	197	73·1
4. M. H.	67	177	244	72·5
	68	176	244	72·1

SUMMARY

1. The errors of estimation of cholesterol in blood plasma by the macroscopic technique are mainly due to incomplete extraction of cholesterol, esterification of free cholesterol in the extracted material and loss of sterol during the extraction following saponification.

2. A modified method is described which avoids these errors. Duplicates of free and ester cholesterol agree within 2% in the majority of cases.

We are indebted to the Government Grants Committee of the Royal Society for a grant in aid of this investigation.

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VI. THE ESTIMATION OF ADRENALINE

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(*Received 3 November 1937*)

References will be found in the paper by Barker *et al.* [1932] to numerous colorimetric methods for estimating adrenaline. Since then other methods have been described by Viale [1933], Kobayashi [1935] and Whitehorn [1935], most of which are applicable only to concentrations >1 μg./ml. and are too unspecific for application to extracts of tissues other than the suprarenals. Whitehorn claimed to estimate adrenaline in blood in a concentration of 0·02 μg./ml. The present paper describes a modification of Whitehorn's method which is simpler and more sensitive.

The method depends on the fact that adrenaline reduces arsenomolybdic acid with the formation of a blue colour. It has been found that brief preliminary treatment of adrenaline with alkali in the presence of oxygen increases the colour considerably. If the treatment with alkali is prolonged the colour is decreased. Adrenaline is known to be oxidized under these conditions, and apparently one of the products of oxidation is a more active reducing agent than adrenaline itself; this product is itself destroyed by longer exposure to alkali. The nature of this product is unknown. A series of substances allied to adrenaline, including adrenalone has been tested, but none is the product in question since none produced as much colour as adrenaline itself. *p*-Sympatol was the only substance which resembled adrenaline in giving increased colour after alkali treatment; it was also the only substance whose side chain was identical with that of adrenaline. These facts suggest that this particular property of the side chain of adrenaline is highly specific, and that if the reducing power of a tissue extract be increased by treatment with alkali the observation can be taken as evidence that the extract contains adrenaline or some other phenol with the same side chain.

Arsenomolybdic acid is reduced by a number of substances besides phenols, such as ascorbic acid, reduced glutathione, eserine, various polyhydroxy and thiol compounds and in general $\Delta^{1,2}$-diols. To eliminate interference by such substances. Whitehorn employed silicic acid which adsorbs the adrenaline, but not the other compounds. He did not, however, succeed in recovering more than 50% of the adsorbed adrenaline, and he multiplied his results by a factor in order to compensate for the loss at this stage. It has been found more convenient to adsorb the adrenaline on a specially prepared aluminium hydroxide, which is afterwards dissolved in the reagents of the test so that the adrenaline is recovered completely. The optimum *p*H for the adsorption of adrenaline is between 8·0 and 8·5; on the acid side of *p*H 7·0 no adsorption takes place. If an extract is first shaken with $Al(OH)_3$ at *p*H 4 glutathione, and possibly other substances, are removed. Ascorbic acid is not adsorbed at either alkaline or acid reactions.

A test has therefore been devised in which the fraction adsorbed at *p*H 8·5, but not at *p*H 4, is heated with alkali and mixed with the arsenomolybdic reagent. This test is probably at least as specific as that described by Whitehorn.

It is simpler and more sensitive, and there is no need to compensate for any loss of adrenaline. In addition a very specific qualitative test is described, which depends on the effect of alkali.

Experimental

Reagents

(1) *Arsenomolybdic acid.* 60 g. of crystalline sodium molybdate and 10 g. of sodium arsenate are dissolved in about 250 ml. of water and filtered. The filter is washed and to the filtrate and combined washings are added 5 ml. of bromine water and water to 500 ml. For use, 100 ml. of this solution are mixed with 8 ml. of conc. H_2SO_4.

(2) *1:1 Sulphuric acid.* A mixture of equal volumes of conc. H_2SO_4 and water.

(3) *Sulphite and sulphurous acid.* 10 g. of $Na_2SO_3,7H_2O$ are dissolved in 50 ml. of water. This must be prepared every 2 days. Before use 2 ml. are added to 14 ml. of 1:1 H_2SO_4. This must be prepared every half hour.

(4) 4% *Sodium hydroxide.*

(5) *Approx.* N *sulphuric acid.*

(6) *Phenolphthalein.* Dissolve 0·1 g. in 100 ml. of $N/100$ NaOH. It is not permissible to use alcohol. This solution will keep about 3 days.

(7) *Aluminium hydroxide.* 25 g. of pure potassium alum are dissolved by heating in about 200 ml. of water and the solution cooled to about 20°. 5 g. of NaOH in 20 ml. water are added slowly with stirring. The precipitate is filtered and washed several times and suspended in about 100 ml. of water, so that it just flows freely from a pipette. Each batch should be soluble in 4% NaOH, and should be tested for its efficiency in adsorbing and liberating adrenaline. Owing to some action of the $Al(OH)_3$ on adrenaline the apparent recovery is usually 110%.

(8) *Standard adrenaline.* A dilution of 1 : 10,000 is prepared in approx. $N/100$ H_2SO_4. This will keep several days. It is diluted to 1 : 10 million, etc. with $N/100$ acid as required. The diluted standard will keep several hours. The stability is improved if a little glycine is added.

Procedure

The solution to be tested, which should contain between 0·1 and 0·5 μg. of adrenaline (though 0·04 μg. can be detected) should be neutral or faintly acid. Blood should be run into an equal volume of 10% trichloroacetic acid as rapidly as possible after removal from the body. If it is kept for a few min. a substance is formed which is indistinguishable in this test from adrenaline. Other tissues are dropped into trichloroacetic acid (at least 1 ml. per g. of tissue) and then cut up finely. After 30 min. the mixture is filtered and the residue is well washed with trichloroacetic acid. The filtrate or a suitable aliquot is placed in a centrifuge tube, 2 drops of phenolphthalein reagent are added and it is then carefully neutralized with 4% NaOH. One drop of N H_2SO_4 is added (approx. pH 4·0), then 2 ml. of the suspension of $Al(OH)_3$. The solution is shaken and centrifuged (3000 r.p.m.) for 2 min. The supernatant fluid is poured into another tube, and 1 ml. $Al(OH)_3$ per 5 ml. of solution and 1 drop of phenolphthalein are added. Caustic soda is now added drop by drop with shaking until the solution is just distinctly pink (pH 8·5). The solution is shaken and centrifuged as before. The supernatant fluid is discarded, and about 3 ml. of water, made just alkaline to phenolphthalein with NaOH, are poured on to the precipitate; it is centrifuged

and the fluid discarded. 2 ml. of water and 0·35 ml. of 4% NaOH are added to the $Al(OH)_3$, which should now go into solution.

The solution is kept for 2 min. and then 2 ml. of the H_2SO_3 reagent are added and the mixture poured into a tube in a boiling water bath which contains 0·7 ml. of the arsenomolybdic acid which has been heating for 5 min. After exactly 5 min. the tube is removed and placed in a beaker of cold water. The volume is made up to 5·5 ml. and the colour estimated in a colorimeter 15–20 min. later. The colour does not fade but remains constant for at least an hour and then tends to increase slowly.

A blank is prepared by taking the same volume of $Al(OH)_3$ suspension as has been used in the test, adding 2 drops of phenolphthalein, making just alkaline with 4% NaOH and centrifuging. The supernatant liquid is discarded, 2 ml. of water, and 0·35 ml. of 4% NaOH are added to the $Al(OH)_3$, then the H_2SO_3, etc. as before.

A standard blue solution is prepared by subjecting 2 ml. of an appropriate solution of adrenaline to the second, but not necessarily the first, adsorption with $Al(OH)_3$ and subsequent treatment with alkali, sulphite and arsenomolybdic acid as described above. A Leitz two-stage colorimeter with a standard grey solution and filter No. 9 was used for comparing the solutions, but no doubt a simpler instrument could be used. The colour due to the blank must be subtracted both from that due to the unknown and that due to the standard.

The curve shown in Fig. 1 shows the relation between the amount of adrenaline and the colour, after subtracting the colour corresponding to the blank test. This curve is approximately linear for small, but not for large, amounts of

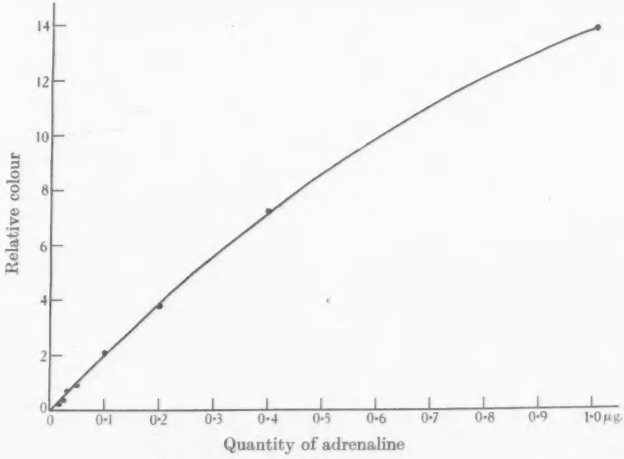


Fig. 1. Colour, in terms of the standard grey solution (using filter No. 9), when adrenaline is subjected to the complete test.

adrenaline. The simplest method of calculating the result of a test would be to read off the amount of adrenaline directly from a curve such as that shown in the figure. This method has been found unreliable because, although constant results have been obtained on any one day, unexplained variations in the position of the curve have occurred when the test has been repeated on different days.

It has therefore been necessary to compare the colour due to unknown solutions with that due to adrenaline solutions treated with the same reagents on the same day. It is found that if the total quantity of adrenaline is less than 0·4 μg. and the ratio of intensities of colour in the two tubes is less than 2, the error introduced by assuming that the colour is proportional to the amount of adrenaline is less than 10%. For some purposes this is accurate enough. Greater accuracy can be obtained by using as standard the colour due to an amount of adrenaline more nearly equal to that contained in the test of the unknown solution, the colour of which is constant for at least an hour.

The smallest absolute amount of adrenaline which can be estimated is about 0·04 μg. and the lowest concentration in which this amount has been estimated was 1 in 5×10^8. The standard deviation of a single observation was estimated as about 0·015 μg.

Specific test

This depends on the increase of colour due to alkali. The solution to be tested is divided into two. One half is treated as above and the other in the same way except that the 0·35 ml. of 4% NaOH which is used to dissolve the $Al(OH)_3$ after the second adsorption is replaced by 0·35 ml. of water containing 1 drop of N H_2SO_4. When the reduction is due to 0·04 μg. or more of adrenaline the ratio of the colour given by the alkali-treated half of the solution to that given by the untreated half is from 2 to 3·5. With smaller quantities the ratio falls.

Table I shows the concentrations of a number of substances required to give a total colour equivalent to that given by adrenaline (1 μg./ml.). These estimates were prepared by testing the solutions of the substance directly without adsorption. The proportional increase of colour when adrenaline was treated with alkali was found to be 5 whereas the ratio was only 3·5 when the two fractions of adrenaline were both adsorbed by the method described above. This difference is apparently due to the fact that the small amount of alkali necessary for adsorption changes the adrenaline slightly so as to increase the colour, thus reducing the apparent effect of the larger quantity of alkali.

Table I also shows that catechol derivatives are active in low concentrations, and that small changes in the side chain abolish the effect of alkali. Sympatol was the only other substance tested which contains the same side chain as adrenaline and the ratio was about the same as that for adrenaline, though it was necessary to employ high concentrations to obtain any appreciable colour. All other substances tested in the series gave ratios of 1 or less. On the other hand, the small amount of colour given in blank tests was found to be increased by alkali in the ratio of 1·1–1·5. Ratios of 2 or over can therefore be used as evidence for the presence of adrenaline.

Results with tissue extracts

Recovery of added adrenaline. Adrenaline was added to rabbits' blood to make a final concentration of 0·1 μg./ml., and it was found, by subtracting the colour due to the blood from the total colour that the recovery was 75–80%. A small loss occurred during the extraction with trichloroacetic acid, since if the adrenaline was added after this stage the recovery was 99%.

Comparison with the biological test. Extracts of suprarenal glands were prepared with trichloroacetic acid, and the adrenaline content was estimated by the test described above and also by comparison of the pressor effect on spinal cats with that of a standard solution of adrenaline. Table II shows satisfactory agreement between the two tests.

Table I. *Showing the specificity of the test*

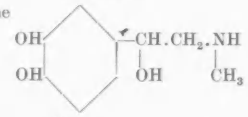
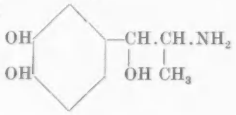
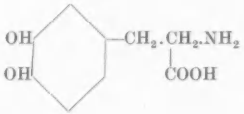
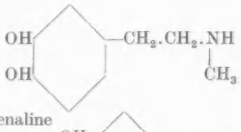
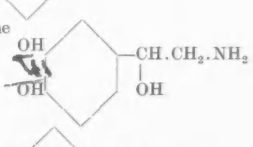
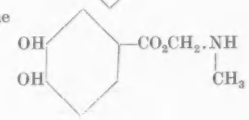
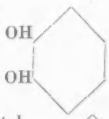
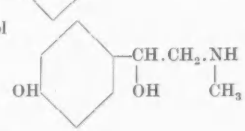
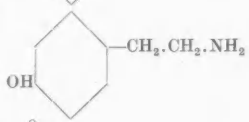
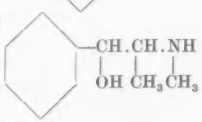
Substance	Concentration (μg./ml.) giving equivalent colour after alkali treatment	Proportional increase of colour due to alkali
Adrenaline 	1	5
Corbasil 	12	No change
Dopa 	12	,,
Epinine 	13	,,
nor-Adrenaline 	16	,,
Adrenalone 	30	,,
Catechol 	100	Diminishes
p-Sympatol 	2000	5
Tyramine 	No colour	—
Ephedrine 	,,	—
Glutathione	500	No change
Ascorbic acid	500	Diminishes
Glyceraldehyde	1000	,,

Table II

Animal	Adrenal content of suprarenal μg./g. Cat blood pressure	Adrenal content of suprarenal μg./g. Colorimetric	Proportional increase due to alkali
Dog	450	400	3·2
Rabbit	150	160	3·1
	250	270	3·4
	600	600	3·4
	1320	1500	—

Other tissues. The results of the application of the test to other tissues are shown in Table III.

Table III

Tissue		Apparent adrenaline content μg./g.	Proportional increase due to alkali
Frog:	heart	0·30, 0·40, 1·0, 1·2	2·5, 2·2
Rabbit:	prostate	0·34, 0·40	2·5, 2·0
	blood	0·050, 0·052, 0·060	1·9, 1·7, 1·5
	intestine	0·033, 0·033	1·0
	heart	0·015, 0·016, 0·017, 0·023, 0·04	1·3, 1·4, 1·5
	liver	0·019, 0·04	1·0
	kidney	0·021, 0·022	1·0, 1·0
	stomach	0·01, 0·019, 0·025	1·0, 1·0
Man:	blood	0·016, 0·020	1·2

The results with frog's heart and rabbit's prostate show rough quantitative agreement with those of Loewi [1936] and Euler [1934] who have found evidence of the presence of adrenaline in these tissues. The increase of colour with alkali confirms the view that the active substance actually is adrenaline.

The other tissues tested certainly did not contain comparable quantities of adrenaline and, in particular, the rabbit's heart contains much less than the frog's heart. In most of the other tissues the blue colour was not increased by alkali and was therefore probably not due to adrenaline. In some cases there was a small increase of colour due to alkali, and though it would, of course, be unwise to assume, without further evidence, that this was due to the presence of adrenaline, this probability is suggested by the results. The presence of such quantities of adrenaline in normal blood serum or plasma appears to be excluded by experiments such as those of Trendelenburg [1923] and Schlossmann [1927].

This fact does not prove that the results obtained in these tests were not due to adrenaline. The blood was collected from the head wound after a rabbit had been killed with a "humane killer" and it is probable that adrenaline is liberated in these conditions. Furthermore, adrenaline passes rapidly into the blood cells [Bain *et al.* 1936] and would therefore be present in extracts of whole blood and not in the serum or plasma.

The results of Table III illustrate the limitations of the method for the estimation of tissue extracts. The amounts of substances other than adrenaline which reduce the arsenomolybdic reagent are equivalent to about 0·01–0·04 μg. of adrenaline per g. of tissue (rabbit liver, intestine, kidney, stomach). Consequently the limit of the method for tissue extracts is probably of the order of 0·1 μg. adrenaline per g.; even results of this order must be interpreted with caution.

SUMMARY

1. A colorimetric method is described which will estimate 0·04 μg. of adrenaline with a standard error of about 0·015 μg.

2. When applied to suprarenal extracts this test gives results agreeing with biological estimates.

3. A specific test is described by which the colour due to adrenaline can be distinguished from that due to other substances.

4. Adrenaline can be estimated in tissues provided that the concentration exceeds 10^{-7}. If the concentration is lower than this, the test gives a result which must be interpreted with caution.

I wish to express my thanks to Prof. J. H. Gaddum for his continued interest and advice throughout the course of this work.

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VII. PREPARATION AND PROPERTIES OF A GLOBULIN PRESENT IN THE ALBUMIN FRACTION OF SERUM

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(*Received 30 November 1937*)

It is now generally accepted that the albumin fraction of blood serum consists of a mixture of proteins and recent chemical and immunological work [Hewitt, 1934; 1936; 1937] has led to the recognition of the presence of crystalbumin, seroglycoid and a globulin in addition to traces of a mucoid. Some characteristic properties of crystalbumin and seroglycoid have been described in previous papers and it is proposed to deal in this communication with the separation of the globulin. This globulin is of interest not only from the point of view of the purification of crystalbumin but also in view of its bearing on the question of the existence of different serum globulins.

Many workers have advanced the view that all globulins have fundamentally the same structure with minor modifications of a physical or extraneous character accounting for the observed divergencies in the behaviour of the different globulin fractions that have been isolated. The diverse properties of euglobulin and pseudoglobulin, for example, have been ascribed to different amounts of lipin adsorbed by the proteins [Chick, 1914], it being assumed that the globulins are otherwise structurally identical. This view is in conformity with the similar amino-acid analysis recorded for the two globulins [Hartley, 1914]. On the other hand it has been found that even after the removal of the lipins the solubilities and optical rotatory powers of euglobulin and pseudoglobulin remained different [Hewitt, 1927]. Various analytical differences between various globulin fractions are described by Lustig & Haas [1931] and differing carbohydrate contents have also been observed [Hewitt, 1934]. Sørensen [1925] regards the globulin fraction as a labile association of euglobulin and pseudoglobulin which dissociates during fractionation processes. The ultracentrifuge has enabled Mutzenbecher & Svedberg [1933] to postulate the existence of different globulins and this has recently been confirmed by Tiselius [1937] using an electrophoretic method. As an example of an opposing point of view serum has been regarded as containing a single protein called "orosin" [Block, 1934].

When a question is open to such divergent views further experimental evidence is obviously desirable and it is possible that eventually different viewpoints may be united. Differences between globulins may in some cases be due to minor modifications such as denaturation, etc., whilst other divergencies may be traced to definite variations in chemical structure. Various globulin fractions have been examined with these objects in view.

Experimental

Horse serum or plasma was the source of the proteins investigated, except in a few experiments referred to later in which ox serum was used. Nitrogen determinations were carried out by the micro-Kjeldahl method and carbohydrate

determinations by the Sørensen & Haugaard [1933] modification of the Tillmans & Philippi [1929] method. For the purposes of calculation it is assumed that the polysaccharide present in serum proteins contains equimolecular amounts of galactose and mannose, and results are quoted in terms of galactose-mannose (g.-m.).

Globulin from the albumin fraction

Serum or plasma was diluted with an equal volume of water, treated with sufficient saturated $(NH_4)_2SO_4$ was added to render the mixture 52% saturated and filtered. Crystalbumin crystallized out when the filtrate was adjusted to *p*H 4·7 by the cautious addition of acetic acid. The amount of precipitate was in some cases increased by the addition of a further small amount of $(NH_4)_2SO_4$. After being kept overnight the precipitate was filtered off, leaving the bulk of the seroglycoid in the filtrate which was discarded. A further small amount of seroglycoid was removed by dissolving the precipitate in water and reprecipitating by cautiously adding $(NH_4)_2SO_4$ until crystallization commenced. The precipitate was filtered off after a short time and the filtrate was again discarded. The precipitate was now dissolved in water and the solution adjusted to *p*H 7·0 by addition of *N* NaOH. To the neutral solution an equal volume of saturated $(NH_4)_2SO_4$ was added. This yielded a copious precipitate, although, before the removal of seroglycoid, all the fraction had been soluble in half-saturated $(NH_4)_2SO_4$. The globulin precipitate was filtered off, leaving the bulk of the crystalbumin in solution. Incidentally it may be mentioned that this process provides an excellent method of preparing pure crystalbumin since one or two crystallizations of the protein in the filtrate yield a very pure specimen.

The globulin precipitate was dissolved in water, reprecipitated by half-saturation with $(NH_4)_2SO_4$, filtered off and the process repeated once or twice. Dialysis to remove salts completed the preparation of the protein. The immediately characteristic property of this fraction is its high carbohydrate content. This is about 5% calculated as galactose-mannose, corresponding to some 7·5% of polysaccharide assuming that this has the constitution galactose-mannose-glucosamine commonly assigned to the serum protein polysaccharides. As will be seen in a later section this polysaccharide content is much higher than has been observed with any other globulin and it differs in other respects. In order to distinguish it therefore it is necessary to find a term which, without prejudice to any consideration of its structure, will suggest briefly its behaviour as a globulin and its high carbohydrate content; it is tentatively suggested that "*globoglycoid*" may be a suitable term.

Globoglycoid has been obtained from normal horse serum, from the blood serum of horses immunized against diphtheria and scarlet fever toxins and from normal ox serum. The amount isolated has been of the order of 1 g. from 1 litre of serum. This amount was of course obtained from the albumin fraction and there is probably a further quantity present in the main globulin fraction. It seems probable that globoglycoid is of general occurrence. Other globulin fractions have been prepared for comparison; these are described in the following section.

Serum globulin fractions

The total globulin precipitate obtained by half-saturation of serum with $(NH_4)_2SO_4$ was purified by dissolving in water and reprecipitating with 50% saturated $(NH_4)_2SO_4$. After several repetitions of this process the globulins were fractionated in various ways, including precipitation with varying concentrations of $(NH_4)_2SO_4$ or NaCl and by isoelectric precipitation. Three main types

of fraction may be distinguished, the pseudoglobulin fractions soluble in distilled water at all *p*H values between 4 and 8, the euglobulin I fractions insoluble in water at *p*H 7 and euglobulin II which is soluble in water at *p*H 7 but insoluble at *p*H 6.

The variations in carbohydrate content of the different fractions are quite considerable as shown in the following table.

Table I. *Carbohydrate contents of various globulin fractions*

g. of galactose-mannose per 100 g. of protein

	Euglobulin I	Euglobulin II	Pseudoglobulin	Globoglycoid
Least soluble	1·8	2·2	1·4	4·6
Intermediate	2·4	2·8	1·7	
Most soluble	2·9	3·6	2·3	

The more soluble fractions tend to have the higher carbohydrate content but in no case is the high value of globoglycoid reached. Apart from differences in precipitability and carbohydrate content the main characteristics of the fractions were as follows. Euglobulin I required a salt concentration of well over 1% before it was dissolved and even then gave a turbid solution; euglobulin II dissolved readily in 0·9% NaCl giving a clear greenish-blue solution; the pseudoglobulin was, of course, soluble in water, whilst globoglycoid although soluble gave a slightly opalescent solution in water.

The antitoxic properties in the sera of horses immunized with diphtheria toxin are carried almost exclusively by the water-soluble globulins and the distribution in the case of a batch of antitoxic sera is shown in Table II.

Table II. *Diphtheria antitoxin distribution*

	Total antitoxin content A.U. $\times 10^4$	Purity of antitoxin A.U. per 1 mg. protein	Flocculation time under standard conditions min.
Pseudoglobulin, sparingly soluble fraction	3·2	5·4	25
Pseudoglobulin, main bulk	5000	20·0	68
Globoglycoid	27·4	5·5	175

It will be seen that only a very small amount of antitoxin is carried by globoglycoid and its purity, in terms of the number of units of antitoxin in a given weight of protein, is little more than a quarter that of the main pseudoglobulin fraction. The rapid flocculation of the sparingly soluble fraction and the slower flocculation of the more soluble fractions have been described in a previous communication [Hewitt, 1934].

Amino-acids

Cystine and tyrosine determinations, carried out as described previously [Hewitt, 1934; 1936; 1937], revealed no great difference between globoglycoid and the other globulin fractions but the tryptophan figures are very characteristic. The tryptophan contents of the globulin fractions ranged from 2 to 3% in conformity with previous figures [Hewitt, 1934; Holiday, 1936] but the value for globoglycoid was much lower, being in the neighbourhood of 0·8%.

This low tryptophan content, less than half that of other globulins, may account for the behaviour of globoglycoid when hydrolysed with acids. The amount of humin formed is much less with globoglycoid than with the other

globulins when heated with mineral acids. It will be recalled that crystalbumin gives no humin when hydrolysed with acids, owing, it was suggested, to the absence of carbohydrate, and it is interesting that globoglycoid despite its high carbohydrate content should yield so little humin. This strengthens the view that tryptophan is of importance in this respect and that tyrosine and cystine play little part.

Discussion

The presence of a globulin in the albumin fraction of serum has been detected immunologically [Hewitt, 1937] and by an electrophoretic method [Tiselius, 1937], and this globulin fraction has now been separated. When examined it is found to exhibit characteristic properties differentiating it from other globulin fractions. It has an extremely high polysaccharide content, probably about 7·5% calculated as galactose-mannose-glucosamine, and to distinguish it from other globulins the name globoglycoid is tentatively suggested. As observed in the case of seroglycoid and ovomucoid the high polysaccharide content tends to confer increased solubility and decreased precipitability compared with other proteins. Globoglycoid requires a higher concentration of $(NH_4)_2SO_4$ to precipitate it than other globulins but it is precipitated by 2% trichloroacetic acid and is coagulated when heated in boiling water, unlike pure seroglycoid or ovomucoid.

A further striking characteristic of globoglycoid is its low tryptophan content which is less than half that of the other globulins. This low tryptophan content is reflected in a decreased tendency to humin formation when heated in acid solution.

In serum from horses immunized with diphtheria toxin only some 0·5% of the antitoxin is found in the globoglycoid and the potency measured in antitoxic units per g. of protein is much less than in the main pseudoglobulin fraction. The phenomenon previously observed [1934] of the rapid flocculation of the antitoxin present in the sparingly soluble pseudoglobulin fractions and the slower flocculation in the more soluble fractions is confirmed but no explanation is forthcoming.

An immediate practical application of the observation of the properties of globoglycoid is the facilitation of the preparation of pure crystalbumin which is frequently required for physico-chemical or immunological experiments.

Incidentally to these observations on globoglycoid some evidence becomes available as to the essential uniformity or multiplicity of the serum globulins. The carbohydrate content of serum globulin was found by Rimington [1931] to be about 2%, by Sørensen & Haugaard [1933] to be 1·8% and by the present author [1934] to range from 1·5 to 3·0% in different fractions (all calculated as galactose-mannose). It is of interest also to note that globulins of different carbohydrate contents are separated by electrophoresis, but Tiselius [1937] states that his figures are of comparative value only. These are 2·2, 0·7 and 0·4% respectively for the β-, γ- and α-globulins. In the present series of observations the carbohydrate contents ranged in the case of pseudoglobulin from 1·4 to 2·3%, the low values being associated with the sparingly soluble fractions, and for the euglobulins from 1·8 to 3·6%.

If it be conceded that the polysaccharide present is an intrinsic part of the protein molecule, and its firm attachment and difficult removal make this conclusion appear probable, then the evidence offered by the different carbohydrate contents seems to show quite definitely that chemically distinct proteins occur in the globulin fraction of serum. The low tryptophan and high carbo-

hydrate contents of globoglycoid suggest that it is different from the main bulk of the globulin fraction.

Doladilhe [1936] has commented on the different physical properties of the euglobulins insoluble in distilled water at *p*H 7 and at *p*H 6 respectively. Difficulties arise in considering all such evidence owing to the susceptibility of proteins to denaturation with consequent modification of properties. Considerable differences in chemical composition, however, can hardly be due to minor denaturation changes, and it is difficult to envisage the range of denaturation effects extending to profound hydrolyses of the protein molecule in the absence of any drastic reagent or *p*H change or any considerable rise in temperature. Nevertheless it is necessary to be wary in accepting evidence of differences between different proteins unless denaturation effects can be definitely excluded, especially when mixtures of proteins are being considered.

It should be emphasized that the term globulin in this paper is used in its customary sense of any protein fraction precipitable by half-saturated $(NH_4)_2SO_4$. This is, of course, an arbitrary classification and may lead to difficulties but its use in this sense is retained in view of its general acceptance.

Summary

1. A globulin fraction tentatively named globoglycoid has been separated from the albumin fraction of blood serum.

2. Removal of globoglycoid facilitates the preparation of pure crystalbumin.

3. Globoglycoid has a high carbohydrate and a low tryptophan content.

4. Both euglobulin and pseudoglobulin are separable into fractions of different carbohydrate content.

5. The evidence available is discussed in relation to the question of the existence of different serum globulins.

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VIII. DECOMPOSITION OF CELLULOSE BY *CYTOPHAGA*. I

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Introduction

The decomposition of cellulose by micro-organism may be brought about by (*a*) aerobic, (*b*) anaerobic, (*c*) thermophilic bacteria or by (*d*) fungi. For a general account of the subject the reader is referred to the works of Thaysen & Bunker [1927], Waksman [1931], Pringsheim [1932] and to the more recent monograph by Khouvine [1934]. In this paper we are only concerned with aerobic decomposition and more particularly with that first described by Hutchinson & Clayton [1919]. To the organism employed, which was isolated from the soil, these authors gave the name *Spirochaeta cytophaga* (n.sp.). The principal features of the changes wrought by this organism are the formation from cellulose of a mucilage of complex structure, together with a yellow pigment. An important point is that cellulose is the only source of carbon which the organism will use. There have been several studies of this and closely related organisms subsequent to the publication of Hutchinson & Clayton, but these have been mainly concerned with the life-cycle, morphology and distribution of the organisms. Doubtless even earlier workers had investigated a similar type of bacterial activity, e.g. van Iterson [1904]. We have concerned ourselves principally with the metabolism of the organism and a preliminary account of the work has already been given [Walker & Warren, 1934]. The work is by no means complete but is being published because one of us has now been obliged to withdraw from participation and because many points have arisen which may take some time to settle.

Identity of organism

The organism used was isolated by us from the local soil and purified after the manner described by Hutchinson & Clayton, which is a dilution method and was further purified by streaking filter paper embedded in silica-gel salt medium, after the manner of Winogradsky [1929]. The simplicity of the medium (inorganic salts + cellulose) constitutes a deterrent to the growth of contaminating bacteria. The metabolic products, too, are not readily susceptible to bacterial attack. It may perhaps be mentioned that our organism has been maintained in artificial culture for more than four years now. The problem of identifying the organism precisely has been rendered more difficult by the divergences of opinion expressed by those who have worked in this field of late years. In general features our organism resembles that described by Hutchinson & Clayton and called by them *S. cytophaga*, although the coccoid form described by them seems to be absent from our culture. Winogradsky in a series of papers culminating in a comprehensive memoir [1929] disapproves the life cycle proposed by Hutchinson & Clayton for their organism and claims that there are many different species of kindred cellulose-decomposing organisms. He examined some dozen different species and for classification purposes he proposed three new genera, *Cytophaga*,

Cellfalcicula and *Cellvibrio*; the particular species is designated by some appropriate suffix. Hutchinson & Clayton's organism appears to be renamed by him *Cytophaga Hutchinsoni*. The important point, and indeed the criterion of membership of this genus, is that the organism should attack cellulose and cellulose only, with the formation of a mucilaginous substance, provisionally called oxycellulose, and a pigment. The pigment is usually yellow and there should be no other metabolic products. The present position is, unfortunately, not quite so clear as when Winogradsky left it. Soon after the latter's contribution, came that of Bokor [1929]; he, too, dismisses the life cycle of *Spirochaeta cytophaga*; in fact, he sweeps away this organism and creates a new one, *Mycococcus cytophaga* (n.sp.), which, metabolically at least, is hard to distinguish from *Spirochaeta cytophaga*. In a brief footnote devoted to Winogradsky's contribution, Bokor expresses disagreement with that author concerning the morphology of the organisms. Since then, Krzemieniewska [1933] has compared *S. cytophaga* (Hutchinson & Clayton) with *Cytophaga Hutchinsoni* (Winogradsky) and comes to the conclusion that they are different and proposes for the former the name *C. myxococcoides*.

All these and ourselves are dealing with what is essentially the same type of organism; i.e. having the same biochemical characteristics which have already been mentioned. Such differences as exist are based on interpretation; in particular, the occurrence of more than one form in a culture raises the question as to whether it represents a stage in the life cycle, or a contaminant or a symbiotic organism. Our own culture shows predominantly the flexuous, filamentous type of organism, which seems to us similar to that described by Hutchinson & Clayton. But it must be admitted that there is invariably present a rod-like form, although this is never conspicuous. We are of opinion that this is another closely related species, probably of the *Cellvibrio* genus (cf. Winogradsky's *Cellvibrio flavescens* and *C. ochracea*). It is certainly not a contaminating organism in the usual sense of the word. Like others, we have attempted to separate the two forms and lately have had some success; if complete, this will form the subject of a later communication. For the time being we will refer to the organism as *Cytophaga Hutchinsoni*, in view of the conflict of opinion recorded above.

Culturing

The organism has been grown on a simple salt medium with cellulose as the sole source of carbon. The medium used was that of Hutchinson & Clayton and had the following composition:

$NaNH_4HPO_4,4H_2O$	2·0 g
KH_2PO_4	1·0
($CaCl_2$	0·1)
$MgSO_4,7H_2O$	0·3
NaCl	0·1
$FeCl_3$	0·01
Water	1000

The *p*H was adjusted finally to 7·5 by the addition of *N* NaOH; usually 5 ml. were required per litre of medium. All the salts used were Kahlbaum's or were specially purified. The water used was distilled in glass from alkaline permanganate. For cellulose, our standard supply has been filter paper (Whatman's No. 1), usually in the amount of 1 g. per 100 ml. medium. We have used other sources of cellulose at various times and these will be referred to in recounting

the results. We have also experimented with the above medium, omitting one constituent at a time and we find that calcium chloride is the only substance that can safely be left out. Since this substance frequently brings about precipitation in the medium we have discarded it from our stock medium. To what extent this constitutes an absolute lack of calcium it is not possible to say; it can only be recorded that both our medium and the cellulose employed fail to give any qualitative reaction for calcium; there can thus only be traces of calcium present. A somewhat similar result has been reported by Rippel & Stoess [1932] for various other micro-organisms.

For general maintenance purposes the above medium is set up in 7 ml. amounts in pyrex test tubes, a strip of filter paper, approximately 6×1 cm., introduced, the tubes plugged with cotton-wool and the whole sterilized by steam heating on three successive days. In the quantitative experiments described later, cellulose was supplied in various forms in the proportion of 1 or 2 g. per 100 ml. medium

A strip of cellulose inoculated in the usual manner shows no obvious change for three days. Thereafter, bright yellow patches develop on the paper above the water line, with most growth at the actual air-water interface. The cellulose, where attacked, is gradually transformed into a glistening, mucilaginous mass, impregnated with yellow pigment. In 7–10 days the strip is "eaten through" at the surface, or transformed into a translucent form which gives this appearance. No decomposition takes place below the surface; consequently the greater part of the cellulose is unattacked. Sub-culturing has been carried out fortnightly, although successful inoculations have been made on occasion at much longer intervals.

*p*H. In some preliminary experiments specially carried out for the purpose, we judged the *p*H limits of growth to be 6·5 and 9·0, with an optimum at 7·5. In the test tube this *p*H does not change appreciably, but on the large scale, when a much greater proportion of cellulose is decomposed, the *p*H is found to have shifted to the neighbourhood of 7·0 by the end of the experiment.

Oxygen. The aerobic nature of the organism is manifest from its growth at the surface of the medium and since it was desired to accelerate the decomposition of the cellulose, it was natural to try the effect of increasing the oxygen supply. Four flasks were set up each containing 100 ml. of medium and 1 g. of cellulose (chopped filter paper). Flask 1 was left un-inoculated, whilst each of the others was inoculated with one drop of an active suspension. Flask 2 had a slow stream of nitrogen bubbled through it, flask 3, a slow stream of oxygen and flask 4 was untreated. Residual cellulose was estimated at the end of 21 days. Table I shows the results.

Table I

Flask	Experiment	Residual cellulose g.	Degree of decomposition %
1	Control	0·97	(3)
2	Nitrogen	0·90	10
3	Oxygen	0·64	36
4	Air	0·84	16

The beneficial effect of oxygen is thus evident. Henceforward it became our usual practice in quantitative experiments to pass oxygen through the culture.

Temperature. All cultures have been grown at 29°.

Large-scale experiments. Both for the purpose of obtaining useful amounts of metabolic products and for greater accuracy in quantitative work most of our experiments have been conducted on a scale of 2 l. of medium, containing 20 g. of cellulose. Such cultures were grown in large, squat pyrex flasks, of 4 l. capacity, and a slow stream of oxygen was bubbled through the medium. The oxygen was supplied from a cylinder and was fed to the culture after passing through a solution of permanganate. The duration of an experiment was usually 28 days. In Table II are recorded the results of various attempts to increase the amount of

Table II

	Proportion of cellulose decomposed
Normal	33%
Halving the cellulose	31
Doubling the ammonium salt	12
Doubling incubation period	38

cellulose decomposed. The normal value in the above table represents the mean value obtained in thirty-two different experiments, recorded later (Table IV). Doubling the nitrogen supply (microcosmic salt) gave an unexpected result; but the matter was not pursued further as we were only interested in increasing the decomposition. Doubling the incubation period naturally had a favourable effect, but not to an extent commensurate with the extra time taken.

Forms of cellulose. Filter paper is the standard type of cellulose which we have used. This is very convenient in test tubes, but less so for flask work because it usually sinks to the bottom of the medium; or, if chopped, adheres to itself or the flask. We have investigated various types of cellulose with a view to finding the most suitable for this kind of work. Eventually we chose cellulose wadding, following the suggestion of Dr R. B. Fisher of this Department. This is easily disintegrated, yet it is of such a texture that it floats readily, a desirable property where aerobic organisms are concerned; we have used it in practically all our experiments. Later, seeking a finer form of cellulose with a view to speeding up the decomposition, good results were obtained using cigarette paper.[1] But it has certain drawbacks; notably, frothing during aeration was excessive and the residual unchanged cellulose was too powdery for convenient handling. Cellophane is readily decomposed by the organism, but suffers from the same drawbacks as chopped filter paper and to a more marked extent. It presents certain interesting points of difference from filter paper cellulose. Thus pigment formation is less conspicuous; and the mucilage formed sinks to the bottom of the medium, whereas ordinarily it floats. Possibly this is a reflection of the difference in micellar structure, or rather the absence of such organized structure in cellophane.

Other forms of cellulose which we have found are attacked by the organism in the usual way are cotton, linen and parchment paper—the latter made by steeping filter paper in 66% sulphuric acid and washing. Cellulose acetate is not attacked. The exclusiveness with which cellulose, and cellulose only, is attacked makes it not inconceivable that the organism could be used for the detection of cellulose.

Other carbon compounds. The failure of the organism to grow on any other carbohydrate was first established by Hutchinson & Clayton and these authors

[1] Kindly obtained for us through the agency of Mr A. E. Wilhelmi.

also tested a number of lower fatty acids (as calcium salts), with negative results. We have been equally unsuccessful in inducing growth on anything but cellulose and slightly hydrolysed cellulose (parchment paper). Any further hydrolysis of the cellulose results in products incapable of sustaining growth.

We have also tried whether the attack on cellulose could be facilitated in any way by the presence of various lower fatty acids. The following were supplied in the form of their sodium salts, in a strength of 0·1 %, along with cellulose: maleic, fumaric, lactic, pyruvic, succinic, citric, tartaric and oxalic. In no case was there any enhanced growth; in the case of oxalic, there was complete inhibition. Other substances which were tried for adjuvant action were furfuraldehyde, furfuryl alcohol and pyromucic acid; all without effect. A partial inhibition was observed with glycerol present in a concentration of 0·3 %.

An important omission from the list of carbohydrates tested by Hutchinson & Clayton is cellobiose. We have tested this and find that alone it is incapable of supporting growth and in the presence of cellulose it exercises an inhibiting effect at a concentration of 1·0 but not at 0·1 %.

We have confirmed the observations of Hutchinson & Clayton that dextrose is inimical to the growth of the organism; we found a marked effect at 0·05 % concentration and almost complete inhibition at 0·1 %. Potassium cyanide, we noted, had a similar effect in low concentration. In both cases, where inhibition was only partial, such growth as took place was at an appreciably higher level above the surface of the medium than usual.

Metabolic products

The principal products of the action of *Cytophaga* upon cellulose are (1) a mucilaginous material, described by Hutchinson & Clayton as akin to pectin and by Winogradsky as oxycellulose, and (2) a pigment, usually yellow, although a variety of colours is obtainable from kindred types, according to Winogradsky. Again, according to the latter, there are no other metabolic products. Hutchinson & Clayton reported small amounts of volatile fatty acids, but these were attributed by Winogradsky to contamination of their cultures. None of these authors makes any mention of carbon dioxide, which is a conspicuous product of our cultures; indeed it accounts for the greater part of the carbon lost. Possibly its production is to be taken for granted. With regard to other products, we have not been able to detect any volatile fatty acids, but have observed traces of what is considered to be higher fatty acid and, in small amount, relatively simple but non-reducing carbohydrates; the latter are probably penultimate degradation products of cellulose. Finally, we have noticed that large scale cultures have a faint but distinct odour, which implies the production of some volatile carbon compound.

For the purpose of studying the metabolic products a considerable number of large-scale experiments has been carried out. The general technique of these has already been described (p. 34). Many variations in the procedure for working up the material have been practised, either for the purpose of increasing the yield or for obtaining a product of greater purity. These are too numerous to mention individually and only the most satisfactory procedures will be described here. The unchanged cellulose is filtered from the culture, using a piece of fine, unbleached linen. It is squeezed as dry as possible and is then thoroughly extracted with hot water, alcohol and ether in turn, after which it is allowed to dry in the air and is subsequently weighed. The alcoholic extracts are reserved for working up the pigment. The filtered culture medium, together with the aqueous expressate, is treated with three times its own volume of 97–99 %

alcohol containing 1 ml. of concentrated hydrochloric acid for every litre of medium to be treated. This brings about the separation of the mucilage. These preliminary operations occupy some 2–3 hr.; the activities of the organism probably cease earlier with the separation of the cellulose and its extraction with hot water. After being allowed to stand for at least 24 hr., the mucilage is obtained by siphoning off the fluid portion and finally by centrifuging. The siphonate is neutralized and allowed to stand a further 24 hr., which results in the separation of most of the salts. It is filtered and concentrated almost to dryness under reduced pressure. The residue is extracted with hot 99 % alcohol, united with the first alcoholic extract of residual cellulose (which carries a good deal of the pigment with it) and filtered. This constitutes the *crude* pigment in alcohol.

Mucilage. The crude mucilage is not easily purified. It is insoluble in water, although it swells up considerably in this medium and retains water of imbibition tenaciously. It is soluble in alkalis, giving a viscous solution, which is difficult to filter and froths readily. We have dissolved the mucilage in dilute ($N/5$–$N/50$) soda or ammonia, using 200–250 ml. for the mucilage from each litre of medium. The ammonia solution seems to be more easily filtered, but even with this salt it is desirable to filter hot, first through linen, then through filter paper. It may be necessary to change the filter paper more than once in order to complete the filtration in a reasonable time. A useful variant is to filter through a thick piece of cellulose wadding. The filtered solution is then acidified with hydrochloric acid, dialysed in cellophane or collodion sacs against distilled water for 7–10 days, separated by siphoning, washed and dehydrated with alcohol. Separation of the mucilage by centrifuging is only effective in an alcoholic medium; in water the mucilage tends to rise to the surface. We have, on occasion, separated it from aqueous medium by saturation with ammonium sulphate, but this has not been a general procedure. Although colourless after the final precipitation with alcohol, the ultimate product gradually darkens and shrivels as it dries and in the end may be almost black. Nevertheless, on soaking in water a sufficient length of time, it will undergo imbibition and assume a colourless, mucilaginous appearance, and if a little alkali be added it will be dispersed to give an opalescent and colourless solution. The amount of mucilage obtained in any given experiment is not large, its bulky appearance when first precipitated being misleading. Under the ordinary conditions of our experiments, quantitative data show that from 1 l. of medium, containing initially 10 g. of cellulose, about 1 g. of mucilage may be expected; in practice, it was appreciably less than this. We have endeavoured to prepare various salts of the mucilage, but these have not been very helpful from the purification point of view, being of variable composition and often very little different from the original mucilage in appearance. It is possible to obtain a white fibrous salt by precipitation with barium salts from alkaline solution, but this has been of variable composition; values of 20–29 % barium content have been obtained, of which the latter is the more reliable. The preparation of a barium salt from acid solution, after the manner of Carré & Haynes [1922] for the calcium salts of pectic acid, yielded a product containing approximately 5 % barium. The use of barium as an agent in the separation and characterization of the mucilage has therefore been discarded. Such analytical data as we possess have been obtained from the free mucilage. Qualitative tests have revealed nothing but the presence of C, H and O; and empirical analysis gave C, 33·3 %, and H, 6 %, O (by difference), 60·7 %; equivalent, 1430. A more highly purified "fraction" (see below) gave C, 40·1 %, H, 6·7 % and O (by difference) 53·2 % and an equivalent of 1107. Too great a

stress must not be laid upon the above figures whilst any uncertainty exists—as it does—as to whether a single substance is under examination or a mixture. Hutchinson & Clayton refer to the possibility that it may represent "a whole range of degradation products"; whilst we ourselves have been able to fractionate the mucilage (i.e. the apparently homogeneous substance precipitated by acid alcohol) and are at present engaged in examining these fractions. It is interesting that one of them should appear to exhibit in an exaggerated degree the frothing tendency characteristic of the whole mucilage fraction. To return to the consideration of the latter as a single substance, there is no doubt that it has many features in which it resembles the pectin group of substances; but quite early we were able to exclude pectin itself by demonstrating that the mucilage contained no methoxyl groups. On the other hand, there is much to be said for the view that it is akin to oxycellulose.

With a view therefore to characterizing the substance, we have carried out estimations of its furfuraldehyde value and its uronic acid content. The former was determined by the method described by Dorée [1933], based on the original Kröber [1900] procedure; the uronic acid by the method of Nanji *et al.* [1925]. The results of these estimations have varied somewhat according to the treatment meted out to the mucilage during purification. The most reliable results are given below in Table III, inset between typical values for pectic acid and oxycellulose culled from the literature and inserted for comparison purposes.

Table III

	Furfural %	Uronic acid %
Pectic acid	20	70
Mucilage	6	14
Oxycellulose (acidic)	1–3	3–12

It would seem that the mucilage is rather more oxidized than oxycellulose. The figures for chemically prepared oxycellulose, however, relate to what is admittedly a mixed product, for it is known that acidic oxycellulose when extracted with alkali reveals a certain proportion of unchanged cellulose. If allowance be made for this fraction, it would bring the figures nearer to those of the mucilage. It may be of interest to note that the uronic acid value for the mucilage would indicate a minimum molecular weight of 1257; whilst the equivalent (p. 36) was found to be 1107–1430.

Hydrolysis of the mucilage has been effected by boiling under a reflux with $N/2$ sulphuric acid, in the first place for 4 hr. and in a second experiment for 12 hr. The solution goes black and there is much humin-like material left even at the end of 12 hr. At the beginning of hydrolysis there is an appreciable evolution of carbon dioxide, indicative of the uronic acid content. The removal of the sulphuric acid quantitatively with baryta reveals that there has been no increase in acidity; there are thus no acids other than uronic in the mucilage molecule. The neutralized hydrolysate was found to reduce Benedict's solution and to give positive reactions for pentose. It was concentrated and submitted to fractionation with alcohol of various strengths. Insufficient material being obtained for characterization of any individual compound, the final fraction was used in making an osazone in the usual way. Recrystallized from water, this osazone had M.P. of 160–161° (uncorr.). This, together with its appearance under the microscope, indicated it to be the osazone of xylose; a finding confirmed by its rotation. No other product was detected. The yield

however is unsatisfactory and it will be necessary to obtain quantitative information concerning the products of hydrolysis before it can be claimed that xylose is the only significant product. When sufficient starting material has been accumulated, other methods of hydrolysis will be attempted. Other workers have failed to obtain reducing substances on hydrolysis and it is clear that this reaction, as with cellulose itself, is not straightforward. We conclude therefore that the mucilage is an oxycellulose, in the sense that this is taken to mean fragmentation of the cellulose chain coupled with oxidation of a certain proportion of primary alcohol to carboxyl groups (in this case 1 in 8).

Pigment. The characterization of the yellow pigment has proved elusive. The following procedure has been adopted for its separation. The alcoholic extract of crude pigment is concentrated by distillation under reduced pressure to approximately 1/10 of its volume. The concentrate is made alkaline with soda, filtered, acidified with hydrochloric acid and extracted with ether. Invariably a small proportion of the pigment remains in the aqueous phase and cannot be extracted by further treatment with ether. The ether extract is dried with anhydrous sodium sulphate, filtered and the ether evaporated. The dark brown viscid material so obtained has a somewhat pungent odour and is acidic in reaction. It is insoluble in water and soluble in all the usual organic solvents. It will dissolve in alkalis giving brown solutions which are colloidal, owing to the presence at this stage of a small amount of higher fatty acid. This latter may be separated from the pigment by solution in methyl alcohol or in acetone, from which there is deposited on cooling a small amount of white material, soluble in light petroleum, and of M.P. approximately 32°; this product forms a colloidal solution in alkalis and gives a lead salts soluble in ether. It is presumed to be a fatty acid, but insufficient material has been available for further investigation. Its removal leaves the properties of the pigment unchanged, except that the latter now gives clear brown solutions in alkalis. A great variety of procedures has been tried with a view to obtaining the pigment (or a derivative of it), in crystalline form, all without success. Similarly, persistent qualitative investigation has yielded nothing tangible. Such evidence as we have suggests that the pigment is an unsaturated aliphatic acid of relatively small molecular dimensions. There is certainly no support for the suggestion of Hutchinson & Clayton that it is a carotenoid pigment. Chemically, the outstanding points to note are the great ease with which it is oxidized by permanganate (acid, alkaline or neutral) and the formation of iodoform on treatment with alkali and iodine.

Miscellaneous products

The most important of these quantitatively is carbon dioxide. As will be seen in the next section, approximately two-thirds of the cellulose which is decomposed appears as carbon dioxide. Apart from a slight initial amount, apparently due to the inoculum, the evolution of carbon dioxide synchronizes with the visible signs of decomposition.

The presence of material judged to be higher fatty acid has already been recorded. The amount is so small as to suggest the possibility that it is a structural constituent rather than metabolic product of the organism. Of volatile fatty acids, as reported in traces by Hutchinson & Clayton, we could find no evidence.

A small amount of some carbohydrate accompanies the pigment in the early stages of its separation; it must therefore be soluble in 75 % alcohol. Preliminary evidence indicates that it is non-reducing, but gives rise to pentose on hydrolysis.

Somewhat similar products can be obtained by fractionation of the crude mucilage. Doubtless they represent late stages in the degradation of cellulose.

Finally, mention should be made of the odour arising from large-scale cultures, such as 2 l. On the small scale, the odour is too slight to detect. This odour is quite distinctive and is an invariable feature in massive cultures. It is difficult to describe; it is not unpleasant to most people, although rather sickly; it recalls the aroma of a stagnant woodland pool. We have not succeeded in finding a suitable vehicle for trapping it and it is produced in extremely small amount in all probability.

It will thus be seen that there are several by-products of metabolism, produced only in small amounts, which it seems desirable to investigate further. In consequence, attention has lately been directed towards increasing the rate of decomposition by the organism, with a view to collecting larger amounts of metabolic material.

Improved method of cultivation

It was obvious that a plentiful supply of oxygen was the most important requirement and various methods of cultivation have been tried with this in mind. Conditions which ensure a plentiful supply of oxygen often minimize the amount of fluid medium, whereas the second desideratum for vigorous growth of *Cytophaga* is that the mucilage formed should be washed away from the unattacked cellulose fibres. We have now succeeded in achieving both these objectives by the use of flasks similar to those devised by Kluyver [1925] but of 2-l. capacity. In these flasks, the air, entering through the medium, is broken up into fine bubbles by means of a sintered glass filter; this ensures saturation of the medium with oxygen and efficient agitation at one and the same time. Using this form of cultivation, we have been able to increase the total decomposition of cellulose in a culture and to shorten its duration (see Table IV). By this means we hope to collect sufficient material for further investigation.

Quantitative results

The following data are supplied as a guide to the fate of the cellulose which disappears under the attack of *Cytophaga*. They do not pretend to give an exact carbon balance sheet, since they were largely accumulated in experiments concerned primarily with the collection of metabolic products. For instance, it was our regular practice, whatever the purpose of the experiment, to determine the amount of cellulose not attacked. The procedure for carrying this out has already been described.

Some idea of the extent to which decomposition takes place may be gained from Table IV, which records the residual cellulose in most of our experiments of a comparable nature.

Experiments omitted from Table IV are concerned with variations in the medium or other conditions.

It will be seen that a general increase in the amount of decomposition was effected through the substitution of cellulose wadding for filter paper. The introduction of cigarette paper resulted in still greater decomposition, but this type of cellulose has drawbacks, which have been mentioned earlier. The table brings out the marked improvement arising from the use of Kluyver type flasks, especially when regard is paid to the duration of such experiments. The lesser degree of decomposition in the case of cellophane (Ex. 69) is due to its texture and greater tendency to sink, with consequent anaerobiosis.

Table IV

Exp. no.	Duration in days	Type of cellulose	Initial amount of cellulose g.	Residual cellulose g.	Cellulose lost g.	Decomposition %
26	23	C.F.P.	20	13·11	6·89	34·45
29	22	C.F.P.	20	13·77	6·23	31·15
32	31	C.F.P.	20	16·24	3·76	18·80
33	28	C.F.P.	20	16·84	3·16	15·80
34	26	C.F.P.	20	17·18	2·82	14·10
35	28	C.F.P.	20	15·68	4·32	21·60
36	30	C.W.	12	7·17	4·83	40·24
38	24	C.W.	20	14·45	5·55	27·75
39	18	C.W.	10	6·88	3·12	31·20
41	30	C.W.	20	15·77	4·23	21·15
42	23	C.W.	20	13·59	6·41	32·05
43	20	C.W.	20	14·58	5·42	27·10
44	20	C.W.	20	14·27	5·73	28·65
45	20	C.W.	20	14·64	5·36	26·80
46	20	C.W.	20	15·33	4·67	23·35
47	49	C.W.	20	12·32	7·68	38·40
48	38	C.W.	20	14·71	5·29	26·45
50	28	C.W.	40	25·48	14·52	36·30
52	37	C.W.	40	28·88	11·12	27·80
54	21	C.W.	40	24·08	15·92	39·80
56	27	C.W.	40	28·00	12·00	30·00
58	28	C.W.	40	21·47	18·53	46·32
60	28	C.W.	40	27·85	12·15	30·40
62	47	C.W.	40	25·87	14·13	35·32
66	27	C.W.	40	25·84	14·16	35·37
67	36	C.P.	20	8·71	11·29	56·45
68	40	C.P.	20	10·46	9·54	47·70
69	40	Ce.	20	13·88	6·12	30·60
72*	10	C.W.	15	7·65	7·35	49·00
73*	21	C.W.	7·5	2·4	5·1	68·00
75*	8	C.P.	5·29	2·62	2·67	50·47
77*	7	C.P.	5·4	2·84	2·56	47·41
					Mean	33·40

C.F.P. = Chopped filter paper. C.W. = Cellulose wadding.
C.P. = Cigarette paper. Ce. = cellophane.
* Kluyver flask.

It is thus apparent that about one-third of the cellulose was decomposed in most of our experiments. With a view to tracing the fate of the cellulose which disappeared, two series of experiments were carried out. In the first of these, the carbon dioxide evolved was measured and is related in Table V to the carbon removed. The carbon dioxide was absorbed in a series of baryta bottles, the contents of which were subsequently titrated; in some experiments aeration was by means of oxygen from a cylinder and in others by suction applied to the Kluyver flask.

Table V

Exp. no.	Duration in days	Initial cellulose g.	Residual cellulose g.	Carbon disappeared g. (A)	Carbon evolved as CO_2 g. (B)	Ratio B/A
1	—	1·0	0·799	0·0893	0·0493	0·552
2	—	1·0	0·661	0·1507	0·0988	0·6557
3	—	1·0	0·6295	0·1646	0·0916	0·5564
4	—	1·0	0·7975	0·09	0·04815	0·535
5	10	15·0	7·653	3·265	2·147	0·6575
6	8	5·407	2·844	1·139	0·775	0·6789

It would seem therefore that the ratio of carbon appearing as carbon dioxide to carbon disappearing is fairly constant and that the carbon dioxide accounts approximately for two-thirds of the cellulose which is decomposed. The remaining third of the carbon lost is accounted for by the mucilage principally and to a lesser extent by the other metabolic products already mentioned. That this is so, we have established by estimating in another series of experiments the carbon in solution, after the manner of Birkinshaw & Raistrick [1931]. The results are given in Table VI.

Table VI

Exp. no.	Duration in days	Initial cellulose g.	Residual cellulose g.	Carbon disappeared g. (A)	Carbon in solution g. (B)	Ratio B/A
35	28	20	15·68	1·92	0·638	0·332
37	28	32	24·679	3·254	1·212	0·372
38/39	22	30	21·328	3·854	1·419	0·3681
41/42	26	40	29·36	4·507	1·589	0·3606
44	20	20	14·27	2·55	0·91	0·3569

It is obvious that these figures, although from a different series of experiments, form the complement to those in Table V. It will also be noticed that there is no significant loss of carbon due to any volatile product; the substance responsible for the aroma can thus arise only in traces. To sum up: two-thirds of the carbon disappearing is in the form of carbon dioxide and one-third remains in solution. This latter is made up of mucilage and other products, and with a view to assessing the relative amounts of each we have attempted the direct measurement of the mucilage. In Table VII below are recorded residual cellulose, carbon dioxide evolved and mucilage formed in each of two experiments. The mucilage was separated as described on p. 36, washed with a little alcohol, dried and weighed. The figures for it given in Table VII are the actual weight of mucilage multiplied by the factor 0·7425, derived from the relative carbon contents of mucilage and cellulose.

Table VII

	Exp. 72 g.	Exp. 78 g.
Initial cellulose	15·0	10·0
Residual cellulose	7·653	6·054
CO_2 as cellulose	4·831	2·618
Mucilage as cellulose	1·782	0·726
Unaccounted for	0·734	0·602

The cellulose not accounted for must be allocated to the formation of the pigment and other metabolic products mentioned in the text as being present only in small amounts.

Discussion and Conclusions

The metabolism of *Cytophaga* presents many interesting points, some of which cannot be discussed here; such are its specific requirement of the most complicated carbohydrate and its simple requirements in all else; its mode of attack on cellulose, particularly the initial reaction; the relation of its activities to those of other micro-organisms in nature. Confining ourselves to the knowledge so far gleaned, we may say that the principal product of the decomposition of cellulose by *Cytophaga* is carbon dioxide, accounting as it does for some two-thirds of the cellulose disappearing. This must be a significant factor in bringing

back into the carbon cycle the immense amounts of cellulose returned to the soil annually. The extent of the decomposition, too, is important, as much as 68% in 21 days and 50% in 8 days having been achieved. It may be contended that this is under laboratory, and therefore optimal, conditions, but the two are not synonymous. Admittedly everything possible is done to facilitate the decomposition, but this may not be as effective as natural processes. Then again the duration of the laboratory experiment is but a fraction of the time available in natural decomposition.

If carbon dioxide is the principal product, it is not the most interesting. It is a big jump chemically from this, the simplest product, to the mucilage. There can be little doubt that this substance should be classed as oxycellulose. This is what Winogradsky suggested, although his evidence was qualitative and he was led to remark that the absence of reducing power constituted an essential difference between biological and chemical oxycelluloses. This distinction does not seem to us to be called for, since oxycellulose is admittedly an indefinite substance. There are, furthermore, two types of oxycellulose generally recognized; an acidic type, having no reducing value and an aldehydic type, having a relatively high copper number. The mucilage has no reducing value and should therefore belong to the acidic type of oxycellulose; this type has a high methylene blue number and does not give a yellow colour with alkalis, features with which the mucilage is in harmony. Nevertheless, it would be safer perhaps to describe it as *an* oxycellulose and more particularly as an oxycellulose formed in alkaline solution. It would seem that the primary alcoholic groups of the cellulose molecule are oxidized to carboxyl throughout the length of the chain; that the consequent instability causes (or renders easier) fragmentation of the large molecule and that the partially oxidized fragments are further attacked by the organism, but that the attack is not carried to completion. To account in this manner for the occurrence of the mucilage implies that any given molecule of cellulose is only partially consumed, which we find easier to believe than that a certain proportion (2/3) of the oxidized cellulose is completely consumed and the remainder not further attacked. It may be remarked here that although the organism seems to require solid cellulose to act upon, micellar structure is not important, as evidenced by the fact that it can utilize cellophane and parchment paper. In discussing here the mucilage as a single entity we are not unmindful that in reality it is probably a mixture, as has been pointed out earlier (p. 37); but in our fractionation the complex acidic polyuronide or oxycellulose predominates and it is this substance we call the mucilage. The second component, and possibly others, are much smaller in amount and may be considered to be later stages in a series of degradations, of which the yield is progressively less and which culminate in carbon dioxide. It is possible that the carbohydrate or polyuronide noted amongst the traces of metabolic products represents some such later stage; whilst the pigment as well may be a mere by-product of the penultimate stage of degradation. The composition of the mucilage is such that the formation of furfuryl derivatives is by no means a remote contingency; the formation of yellow pigment and the slight aroma are also not incompatible with some connexion with furfuraldehyde. We have often searched for evidence for the existence of furfuryl derivatives in the various metabolic products, but such evidence, if occasionally suggestive, has been on the whole negative.

There remains one further point to which attention should be directed. It is our general experience that the mucilage is not readily attacked by bacteria, although it does succumb to moulds. This resistance to bacterial decomposition, together with its great capacity for holding water, suggests that it might well be

one of the "organic colloids" of the soil. Most workers who have handled the material are impressed with its resemblance to humic substances and its location, mode of formation and general properties fit in well with such a role.

Summary

1. The metabolism of the aerobic, cellulose-decomposing organism, *Cytophaga Hutchinsoni* has been studied and the cultivation of the organism described.

2. Of the cellulose decomposed by the organism, it is shown that two-thirds appear as carbon dioxide and one-third principally as mucilage, together with small amounts of other products.

3. Evidence is presented which supports the view that the mucilage is to be considered as an oxycellulose of the acidic, non-reducing type. Xylose has been identified amongst its hydrolysis products.

4. The yellow pigment formed is considered to be an unsaturated aliphatic acid, of relatively small molecular dimensions, and is not, as stated in the literature, a carotenoid pigment.

5. Other metabolic products noted, but in traces only, are: a higher fatty acid, a carbohydrate soluble in 75% alcohol and an unidentified constituent responsible for a slight aroma. No evidence was obtained for the existence of volatile fatty acids.

6. By means of a new technique, ensuring luxuriant oxygenation, it is possible to achieve a 50% decomposition of cellulose in about eight days.

7. Attention is drawn to the biological significance of this type of decomposition.

The authors are grateful to Prof. R. A. Peters for his interest in this work.

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IX. NOTE ON CERTAIN CARBOHYDRATE CONSTITUENTS OF THE BARK OF ASH (*FRAXINUS EXCELSIOR*)

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In the course of an investigation into the nutrition of the ash-bark beetle (*Hylesinus fraxini* Panz.), the results of which are recorded elsewhere [Hopf, 1937; 1938], it became necessary to examine the main carbohydrate constituents of ash-bark, particularly the hemicelluloses. In view of the fact that little has been published concerning the polyuronides of any type of bark, opportunity is taken briefly to record the main results of this investigation.

Experimental

The material used was obtained from branches of ash, about 12 years old, grown at Southwater, Sussex, and cut in winter. The bark was peeled off, and ground to pass a sieve of 60 meshes per inch. Using 50–60 g. of bark, the following fractions were prepared.

(1) Fraction soluble in hot water, obtained by treating the bark five successive times for 6 hr. with water at 90°.

(2) Pectic substances, extracted by 0·5% ammonium oxalate at 90°.

(3) Hemicelluloses, obtained by exhaustive extraction with 4% NaOH in the cold. The extract was further fractionated into hemicelluloses A, B and C by the method of Norris & Preece [1930], but no attempt was made to fractionate further by means of the copper compounds.

For quantitative estimations of the various fractions, portions of 5–10 g. of the bark were similarly treated; the figures given below for yields of various fractions were obtained thus.

Hot water-soluble fraction. The extract contained free sugars, glucosides etc., and gave on addition of excess of alcohol a bulky precipitate mainly of carbohydrate nature, corresponding to 8% of the bark. This product has not yet been investigated thoroughly, but has been found to yield some galactose on hydrolysis, but neither glucose or fructose. Had starch been present in the bark, it would have appeared here [cf. Campbell, 1935], but on no occasion was any starch detected. Staining tests on the bark of growing trees gave positive results for starch, but this disappeared within a short time of felling; the disappearance of starch from wood has been recorded by Wilson [1935] and others.

The properties of this fraction were found to vary somewhat in different samples. Those prepared from recently cut branches gave no colour reaction for pentoses, while other samples, prepared from wood which had been lying in the laboratory for some weeks, gave a definitely positive reaction. Whether this difference was due to some alteration in the physical condition of the wood, allowing of an easier extraction of some pentosan substance, or whether there had actually been a development of pentosan from some precursor, cannot be stated. Chemical changes do occur in wood after felling, while respiration is still proceeding, and research on this subject is needed.

Pectic substances. Addition of excess of acidified alcohol to the ammonium oxalate extract gave a bulky gelatinous precipitate; after washing with alcohol and drying, a product was obtained whose calcium pectate value was 95%, and which was evidently almost pure pectin. The uronic anhydride content of the product was 80%, and the yield (as Ca pectate) was 7% of the bark.

Hemicelluloses. These were obtained as light brown powders when dry. Determinations of furfuraldehyde and CO_2 yield (on distillation with 12% HCl) were made, and the approximate proportions of pentosan, uronic anhydride and hexosan in the hemicelluloses calculated therefrom. The results are given in Table I.

Table I

Fraction	A	B	C
Pentosan (% of fraction)	95	18	15
Uronic anhydride (% of fraction)	—	17	20
Hexosan (by difference)	(5)	65	65
Yield (% of bark)	2·1	13·6	3·1

The individual fractions B and C were hydrolysed and an examination was made of the sugars, etc. in the mixture; lack of material prevented investigation of fraction A, which corresponded to an almost pure pentosan. The constituents whose presence was established were:

hemicellulose B: arabinose, galactose, mannose, galacturonic acid;

hemicellulose C: arabinose, galactose, trace of mannose.

In fraction B, the mannose was estimated at approximately 7% (weighed as phenylhydrazone), and the arabinose 9% (diphenylhydrazone). In view of the fact that in fraction B the total pentosan was of the order of 20%, xylose may have been present, but this could not be confirmed. Fraction C gave a much higher yield of arabinose diphenylhydrazone, and was probably free from xylose; in any event, in such mixtures of sugars the estimations are to be regarded as only very approximate.

Other carbohydrates. An estimation of cellulose in the bark, by the Cross and Bevan method, indicated the presence of 32·5% of cellulose; this cellulose yielded furfuraldehyde corresponding to 7·9% of xylan (i.e. 2·6% of the original bark). The bark was found to contain also 16·5% of lignin.

Discussion

The amount of pectin in the bark was unexpectedly high, being of the order found in leaves rather than in wood [see Nanji & Norman, 1928; Buston, 1935]; the sapwood of ash has been found to contain less than 1% of total pectins.

Again, the hemicelluloses were found to be predominantly of the hexosan-uronide type containing much galactose, whereas it is well known that the hemicelluloses of timber are mainly pentosans, and especially xylan [Norman, 1937, p. 52]. The ash-bark hemicelluloses in fact resemble rather the galacto-arabans isolated from leaves and other relatively non-lignified parts of plants [Buston, 1935]. Comparative figures are very scanty, but the following are typical of those which have been published.

Table II. *Constituents of hemicelluloses B and C*

Material	Pentosan %	Uronic anhydride %	Hexosan %
Bean leaf [Buston, 1935]	22·4	19·6	58
Ash bark (this work)	18–15	17–20	65
Box wood [Preece, 1931]	81	17	—

SUMMARY

Ash bark contains approximately 7% of pectic substances, and 20% of hemicelluloses; in the latter mannose, galactose, galacturonic acid and arabinose have been identified. Hexosan units, especially galactans, are predominant, and in this respect the products from ash-bark resemble those from leaves rather than those hitherto prepared from timber (sapwood).

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Note added 24 December, 1937. Hemicelluloses have been prepared from the sapwood of the timber used in the above work. Xylose was found to be the chief sugar present, accounting for some 60% of the total hemicelluloses.

X. THE ACTION OF THE CINCHONA AND CERTAIN OTHER ALKALOIDS IN BIRD MALARIA. III

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In the first paper [Goodson *et al.* 1930] of this series, it was shown that in a homologous series of alkylquitenines there is a slow and irregular rise in anti-malarial activity as the series is ascended, to a maximum in the neighbourhood of *n*-butyl- or *n*-amyl-quitenine and after that a fall. As the alkylquitenines are easily hydrolysed, it was suspected that some of the irregularities were due to this factor, and it was considered desirable to investigate a homologous series of more stable derivatives of the cinchona alkaloids. For that purpose the alkyl ethers of *apo*quinine and *apo*quinidine were selected. These *apo*-bases proved not to be simple substances, but complex mixtures of quinine and quinidine transformation products of unknown constitution. The results of their chemical investigation have been dealt with elsewhere [Henry *et al.* 1934, 1935, 1937], and a list of the substances isolated in the course of this work is given in Table I with references to the formulae (II to V) printed below, by which they are represented and which will be sufficient to indicate their relationships to each other and to the parent substances, quinine and quinidine, which are stereoisomerides represented by (I: R = Me). The original objective of the investigation has been realized by the preparation of an extensive series of ethers of the principal phenolic base, *apo*quinine (II: R = H), obtained, and of its reduction product dihydrocupreine (VI: R = H), and the opportunity has been taken to test biologically (*a*) all the products mentioned in Table I, which could be obtained in

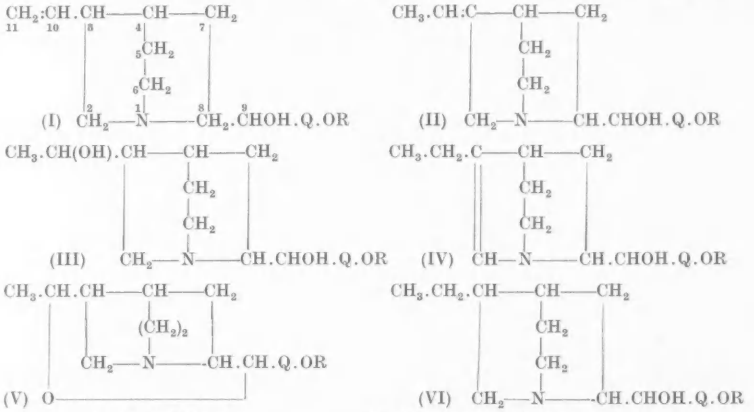
Table I

Name of substance	Formula	Name of substance	Formula
*apo*Quinine	II: R = H	α-*iso*Quinidine†	V: R = Me
β-*iso*Quinine	II: R = Me	γ-*iso*Quinidine†	V: R = Me
*isoapo*Quinine	II: R = H	*apo*Quinidine	II: R = H
α-*iso*Quinine	II: R = Me	*apo*Quinidine methyl ether	II: R = Me
α-Hydroxydihydro*apo*quinine	III: R = H	*neoapo*Quinidine	II: R = H
α-Hydroxydihydroquinine	III: R = Me	*neoiso*Quinidine	II: R = Me
β-Hydroxydihydro*apo*quinine*	III: R = H	α-Hydroxydihydro*apo*quinidine	III: R = H
ψ-Quinidine†	IV: R = Me	α-Hydroxydihydroquinidine	III: R = Me
*isoapo*Quinidine	V: R = H	β-Hydroxydihydro*apo*quinidine	III: R = H
β-*iso*Quinidine	V: R = Me	β-Hydroxydihydroquinidine	III: R = Me

* Corresponding methyl ether unknown. † Corresponding phenolic base unknown.

sufficient quantity, and (*b*) certain interesting by-products arising out of this work, of which the following brief description may be given to indicate their

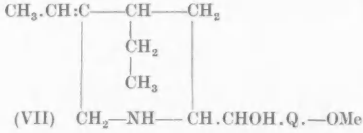
relationship to the main enquiry. It will be clear that formula II requires the existence of two geometrical isomerides represented by *apo*quinine (methyl ether = β-*iso*quinine) and *isoapo*quinine (methyl ether = α-*iso*quinine) in the



Q = Quinolyl residue. R = Alkyl group or hydrogen.

case of quinine. On hydrogenation of either α- or β-*iso*quinine there will be produced dihydroquinine (VI: R = Me) and its epimeride about carbon atom 3, which may be named *epi*-C^3-dihydroquinine. This substance and the analogous *epi*-C^3-dihydroquinidine have been isolated in a pure state [Henry *et al.* 1937] and tested biologically.

In certain of the methods used for the preparation of the crude mixture until recently known as "apoquinine" there is always formed a minute amount of niquine. This base was first obtained forty-five years ago by Skraup [1892], as a well-defined, crystalline substance, but it is still uncertain whether it should be represented by the formula $C_{19}H_{24}O_2N_2$, as proposed by Skraup [1892], by $C_{20}H_{26}O_2N_2$, as suggested by Léger [1937] or by $C_{20}H_{24}O_2N_2$ as adopted by Reyman and Suszko [1935]. Léger has suggested that niquine should be represented by formula (VII) and as a substance of that formula in which the



bridged ring has been converted into a substituted piperidine ring is of obvious biological interest, in the present connexion, specimens of niquine and its reduction product, dihydroniquine, have been made and tested. The corresponding quinidine product, niquidine, first obtained by Domanski & Suszko [1935] and for which these authors appear to prefer the formula $C_{20}H_{24}O_2N_2$, though their analytical results are in better agreement with $C_{19}H_{24}O_2N_2$, has also been prepared together with a new isomeride, which it is proposed to call *iso*niquidine. Niquidine and *iso*niquidine yield the same dihydro-base and, there being no doubt of their chemical individualities, have also been tested biologically (Table IV).

apo*Quinine ethers* (*II*: *R* = *Alkyl*)

*β-iso*Quinine was first shown to be *apo*quinine methyl ether by Suszko [1925]. *apo*Quinine ethyl ether has been described elsewhere [Henry *et al.* 1934; 1935] and attention has been directed to its pneumococcicidal action by various workers [Miura and Okamoto, 1930; Gundel and Seitz, 1933; Maclachlan *et al.* 1934]. The propyl-, *iso*propyl-, butyl-, *iso*butyl-, *iso*amyl-, hexyl-, octyl- and *iso*octyl-ethers were made in Japan or in Germany for bactericidal tests [Ishizaka *et al.* 1933; Liebetruth *et al.* 1935], but were not fully characterized, the German workers describing the bases as amorphous, whereas the present authors found most of them to be well-crystallized substances.[1] In the descriptions given below the names of ethers not previously prepared are marked *. The names of new derivatives of known ethers are printed in italics. The following methods of preparation have been used. Equimolecular quantities of *apo*quinine purified as described [Henry & Solomon, 1934] and KOH were dissolved in alcohol (3 parts), or alcohol and ether when necessary, the solution cooled to 0° and treated with 1 mol. of the alkylating agent. The mixture was kept, at first in a refrigerator, and later at room temperature, until inorganic salt ceased to deposit, usually in 1 to 4 weeks. The brown liquid was decanted, evaporated to dryness in a vacuous desiccator, the residue dissolved in dilute mineral acid, the solution washed with ether and the base recovered by making alkaline and extracting with ether. The higher alkyl ethers were insoluble in dilute mineral acids and in these cases the solid residues had to be washed with dilute acid and ether, and finally with alkali and ether. The ethereal extracts were dried over anhydrous Na_2SO_4, decolored by boiling with charcoal and evaporated, leaving a residue, usually crystalline, which was purified by recrystallization as the base or as a salt. The yields varied from about 30 to 48% except for undecyl and cetyl ethers where they rose to 66 and 68% respectively.

The ethers are very soluble in alcohol, readily so in ether, less so in acetone and sparingly soluble or insoluble in petroleum. The higher members tend to soften and melt indefinitely; their neutral salts with mineral acids are insoluble or sparingly soluble in water, but readily soluble in alcohol; the acid salts are more soluble in water except in the case of the highest members, where the ethanesulphonates had to be used for polarimetric work and for the biological tests.

In the following descriptions the specific rotations are recorded for salts in *M*/40 solution in water, and for the anhydrous base in *M*/40 solution in *N*/10 H_2SO_4, except in the cases where *N*/10-ethanesulphonic acid is specified for the reason already given, and in other instances where the special conditions are stated. The analytical results are recorded for the substance dried in a vacuum, usually at 110°, but at a suitable lower temperature for low-melting products.

n-*Propyl ether*. Amorphous, colourless, precipitate, $[\alpha]_D^{15°}$ −286·8°; *hydrochloride*, colourless needles from acetone, M.P. 249–251° (*decomp.*) with some previous sintering, $[\alpha]_D^{15°}$ −172°. (Found: Cl, 9·15%. $C_{22}H_{28}O_2N_2.HCl$ requires Cl, 9·1%.)

iso *Propyl ether*. Colourless, amorphous precipitate or jelly from solvents, $[\alpha]_D^{15°}$ −288°; *hydrochloride*, colourless needles from acetone, sintering at 240°, M.P. 254–257° (*decomp.*). (Found: Cl, 9·20%. $C_{22}H_{28}O_2N_2.HCl$ requires Cl, 9·1%.)

n-*Butyl ether*. Minute glistening, colourless needles from ether, M.P. 162–164°, $[\alpha]_D^{15°}$ −280·7°. (Found: C, 75·4; H, 8·35; N, 7·7%. $C_{22}H_{30}O_2N_2$ requires C, 75·4; H, 8·25; N, 7·65%); *hydro-*

[1] While this paper was in the press ~~Ritchie~~ Butler *et al.* [1937] published descriptions of a few of the lower members of the series.

chloride, glistening, colourless, felted needles, sintering at 192°, M.P. 219° (*decomp.*). (Found: Cl, 8·9%. $C_{23}H_{30}O_2N_2$.HCl requires Cl, 8·8%.)

n-*Amyl ether**. Glistening, colourless rosettes of needles from ether, M.P. 143–145·5°, $[\alpha]_D^{15°}$ −268·1°; *hydrochloride*, minute, colourless needles from water, M.P. 234·5–236·5° (*decomp.*). (Found: loss on drying, 3·1; Cl, 8·17%. $C_{24}H_{32}O_2N_2$.HCl requires Cl, 8·51%.)

iso *Amyl ether*. Colourless needles, from a mixture of benzene and light petroleum, sintering from 168°, M.P. 185°; $[\alpha]_D^{15°}$ −269·7°; *hydrochloride*, rosettes of minute needles from acetone, M.P. 201–205°. (Found: Cl, 8·5%. $C_{24}H_{32}O_2N_2$.HCl requires Cl, 8·5%.)

n-*Hexyl ether*. Felted mass of glistening short needles from acetone, M.P. 135–137°, $[\alpha]_D^{15°}$ −273·4° (0·1 *N* $EtSO_3H$); *hydrochloride*, colourless, concentrically grouped needles, M.P. 223–225°. (Found: Cl, 7·9%. $C_{25}H_{34}O_2N_2$.HCl requires Cl, 8·2%.)

n-*Heptyl ether**. Long silky needles from acetone, M.P. 137–140°, $[\alpha]_D$ −263·5° (0·1 *N* $EtSO_3H$); *hydrochloride*, crystalline mass from acetone, M.P. 172–176°. (Found: loss on drying, 4·6; Cl, 8·1%. $C_{26}H_{32}O_2N_2$.HCl, H_2O requires for $1H_2O$, 3·9, and for dried salt Cl, 8·0%.)

n-*Octyl ether*. Rosettes of colourless needles from ether, M.P. 141–143°, $[\alpha]_D^{15°}$ −232·5° (0·1 *N* $EtSO_3H$). (Found: C, 76·7; H, 9·1; N, 6·7%. $C_{27}H_{38}O_2N_2$ requires C, 76·7; H, 9·1; N, 6·6%); *hydrochloride*, minute, hard granules, melts indefinitely 90–145°. (Found: Cl, 7·2%. B.HCl requires Cl, 7·7%.)

sec-*Octyl ether**. Amorphous, $[\alpha]_D^{15°}$ −225·6° (0·1 *N* $EtSO_3H$). (Found: C, 76·3; H, 8·9; N, 6·7%. $C_{27}H_{38}O_2N_2$ requires C, 76·7; H, 9·1; N, 6·6%). It yields a well-defined, but abnormal *acid oxalate*, crystallizing in short cream-coloured needles, softening at 70° and frothing at 120°. (Found: C, 62·7; H, 7·26; N, 5·0%. B.$2H_2C_2O_4$ requires C, 61·8; H, 7·0; N, 4·6 and $B_4.7H_2C_2O_4$ requires C, 62·4; H, 7·1; N, 4·7%.)

n-*Nonyl ether**. Colourless, concentrically grouped needles, M.P. 138–141°, $[\alpha]_D$ −211·6° (0·1 *N* $EtSO_3H$) from ether. The *hydrochloride* is a nearly white powder consisting of minute needles from a mixture of benzene and petroleum, M.P. 100–120°. (Found: N, 5·9; Cl, 8·0%. $C_{28}H_{40}O_2N_2$.HCl requires N, 5·9; Cl, 7·5%.)

n-*Decyl ether**. Glistening mass of rosettes of needles from ether, M.P. 124–127°, with some sintering at 120°, $[\alpha]_D^{15°}$ −177·6° (0·1 *N* $EtSO_3H$); *hydrochloride*, minute, colourless needles from a mixture of benzene and light petroleum, M.P. 90–115°. (Found: C, 71·0; H, 8·6; N, 5·8; Cl, 7·4%. $C_{29}H_{42}O_2N_2$.HCl requires C, 71·5; H, 8·9; N, 5·8; Cl, 7·3%).

n-*Undecyl ether**. Colourless, silky mass of rosettes of needles from ether, M.P. 124–129°, $[\alpha]_D^{15°}$ −164·6° (0·1 *N* $EtSO_3H$); *hydrochloride*, colourless, short needles from a mixture of benzene and petroleum, M.P. 100–120°. (Found: C, 71·1; H, 8·9; N, 5·6; Cl, 7·1%. $C_{30}H_{44}O_2N_2$.HCl requires C, 71·9; H, 9·1; N, 5·6; Cl, 7·1%.)

*Cetyl ether**. Rosettes of colourless needles, M.P. 103–106°, $[\alpha]_D^{15°}$ −133·7° (0·1 *N* $EtSO_3H$) from ether; *hydrochloride*, translucent, waxy masses of minute rosettes of needles, M.P. 90–110°, from a mixture of benzene and petroleum. (Found: C, 73·6; H, 9·75; N, 4·8; Cl, 6·3%. $C_{35}H_{54}O_2N_2$.HCl requires C, 73·55; H, 9·7; N, 4·9; Cl, 6·2%.)

isoapo*Quinidine alkyl ethers* (*V*: *R*=*Alkyl*). Of the three *apo*quinidines of this type only *isoapo*quinidine was available in quantity and from this a series of ethers was prepared by the general method already described. The yields ranged from 20 to 27%: the products were usually amorphous, but yielded crystalline salts, of which the acid sulphate, $B.H_2SO_4$, was generally the most convenient for purification. The methyl ether is the well-known *β-iso*quinidine.

*Ethyl ether**. Amorphous. The *neutral sulphate* crystallizes from water in prisms, M.P. 84° or 140–145° (dry), $[\alpha]_D^{15°}$ −43·4°. (Found: loss on drying, 15·3; C, 64·7; H, 7·35; N, 7·2; S, 4·0; EtO, 11·6%. Calc. for $10H_2O$: loss on drying 15·7%; $(C_{21}H_{26}O_2N_2)_2.H_2SO_4$ requires C, 65·1; H, 7·0; N, 7·2; S, 4·1; EtO, 11·6%.)

n-*Amyl ether**. Amorphous. The *acid sulphate* forms needles from 80% alcohol, M.P. 173°, $[\alpha]_D^{20°}$ +26·2°. (Found: loss on drying, 3·8; C, 60·45; H, 7·3; N, 5·6; S, 6·3%. Calc. for $1H_2O$, loss on drying, 3·6%. $C_{24}H_{32}O_2N_2.H_2SO_4$ requires C, 60·2; H, 7·2; N, 5·85; S, 6·7%.)

iso*Amyl ether**. Amorphous. The *neutral sulphate* separated from 50% alcohol in needles, sintering at 100°, M.P. 130° (*decomp.*), $[\alpha]_D^{20°}$ +31·7° (0·1 *N* H_2SO_4). (Found: loss on drying, 9·1; C, 67·2; H, 7·7; N, 6·7; S, 4·0%. Calc. for $5H_2O$ loss on drying, 9·2%. $(C_{24}H_{32}O_2N_2)_2.H_2SO_4$, requires C, 67·1; H, 7·7; N, 6·5; S, 3·7%.)

n-*Hexyl ether**. Amorphous. The *acid sulphate* crystallized from 80% alcohol in needles, M.P. 175°, $[\alpha]_D^{20°}$ +24·9° (0·1 *N* HCl). (Found: loss on drying, 4·0; C, 59·5; H, 7·6; N, 5·8; S, 6·8%. Calc. for $1H_2O$, loss on drying, 3·5%. $C_{25}H_{34}O_2N_2.H_2SO_4$ requires C, 60·9; H, 7·4; N, 5·7; S, 6·5%.) This salt gave consistently low figures for C ranging from 59·4 to 59·6%.

n-*Heptyl ether**. Amorphous. The *acid sulphate* separated from 85% alcohol in needles, M.P. 170°, $[\alpha]_D^{20°}$ +25·3° (0·1 *N* HCl). (Found: loss on drying, 3·4; C, 61·1; H, 7·5; N, 5·5; S, 6·1. Calc. for $1H_2O$, loss on drying, 3·4%. $C_{26}H_{36}O_2N_2.H_2SO_4$ requires C, 61·6; H, 7·6; N, 5·5; S, 6·3%.)

n-*Octyl ether**. Amorphous. The *acid sulphate* formed needles from 95% alcohol, M.P. 161°, $[\alpha]_D^{20°}$ −5·1° (0·1 *N* HCl). (Found: loss on drying, 3·7; C, 61·9; H, 7·75; N, 5·3; S, 6·2%. Calc. for $1H_2O$, loss on drying, 3·3; $C_{27}H_{38}O_2N_2.H_2SO_4$ requires C, 62·3; H, 7·75; N, 5·4; S, 6·2%.)

Alkyl ethers of dihydrocupreine and dihydrocupreidine (VI: R = Alkyl).

When dihydroquinine or dihydroquinidine (VI: R = Me) is demethylated no intramolecular change occurs, such as takes place in the demethylation of quinine or quinidine, and the corresponding phenolic base, dihydrocupreine or dihydrocupreidine is formed. The former is one of the hydrogenation products of *apo*quinine or its geometrical isomeride, *isoapo*quinine (II: R = H) due to saturation of the ethylenic linkage between C^3 and C^{10}, and the same relation holds for the hydrogenation of *apo*quinidine (II: R = H) to dihydrocupreidine (VI: R = H). In view of the quantitative change in antimalarial activity induced by the hydrogenation of quinine to dihydroquinine and of quinidine to dihydroquinidine [Buttle *et al.* 1934], it was of interest to compare the antimalarial activities of the alkyl ethers of the *apo*-bases with alkyl ethers of their hydrogenation products—the alkyl dihydro-cupreines and -cupreidines. The whole series of alkyl ethers of dihydrocupreine from C_2 to C_{16} has been prepared and used for bactericidal, trypanocidal and other biological tests [Morgenroth (with Halberstaedter, 1910; 1911); (with Levy, 1911); (with Tugendreich, 1916): Schaeffer, 1917; (with Braun, 1917)], but only a few members of the series have been characterized and described. The ethyl and propyl ethers were patented [Zimmer, 1912] and the former was described by Giemsa & Halberkann [1918] and by Heidelberger & Jacobs [1919], who also prepared the *sec*-octyl ether. These three, together with the *n*-butyl, *iso*amyl and hexyl ethers were also prepared by Slotta & Behnisch [1933]. The others, described below, are new so far as chemical literature is concerned.

iso*Propyl ether**. Amorphous, $[\alpha]_D$ −226·0°; *hydrochloride*, colourless, hemispherical rosettes of needles from acetone, M.P. 227–230°, with previous sintering. (Found: C, 67·9; H, 8·0; N, 6·8; Cl, 8·6%. $C_{22}H_{30}O_2N_2.HCl$ requires C, 67·6; H, 8·0; N, 7·2; Cl, 9·1%.)

n-*Amyl ether**. Colourless, felted masses of minute needles from acetone, sintering at 117°, M.P. 130–132°, $[\alpha]_D^{15°}$ −210·2°; *dihydrochloride*, needles from water, sinters at 233°, M.P. 240–243° (*decomp.*), $[\alpha]_D^{15°}$ −177·9°. (Found: loss on drying, 4·05; Cl, 15·7%. Calc. for $1H_2O$, loss on drying, 3·8%. $C_{28}H_{34}O_2N_2.2HCl$ requires Cl, 15·6%.)

n-*Heptyl ether**. Colourless needles with $1H_2O$ from acetone, M.P. 130–132°; $[\alpha]_D^{15°}$ −198·9° (0·1 *N* $EtSO_3H$); *dihydrochloride*, sheaves of felted needles, M.P. 140–160° from water. (Found: loss on drying, 8·9; Cl, 14·7%. Calc. for $2·5H_2O$, loss on drying, 8·5%. $C_{26}H_{38}O_2N_2.2HCl$ requires Cl, 14·7%.)

n-*Octyl ether**. Rosettes of colourless needles from acetone, M.P. 119°, $[\alpha]_D^{15°}$ −186·3° (0·1 *N* $EtSO_3H$). (Found: loss on drying, 3·3; C, 76·1; H, 9·4; N, 6·7%. Calc. for $0·75H_2O$, loss on drying, 3·1%. $C_{27}H_{40}O_2N$ requires C, 76·35; H, 9·5; N, 6·6%); *dihydrochloride*, rosettes of needles from

water, M.P. indefinite, sintering from 135°. (Found: loss on drying, 9·4; Cl, 13·7%. Calc. for $3H_2O$, loss on drying, 9·8%. $C_{27}H_{40}O_2N_2.2HCl$ requires Cl, 13·7%.)

n-*Nonyl ether**. Feathery crystals from light petroleum, sintering at 96°, translucent at 102–104°, clear oil at 130°, $[\alpha]_D^{15°}$ −146° (0·1 *N* $EtSO_3H$). (Found: C, 76·8; H, 9·6; N, 6·4%. $C_{28}H_{42}O_2N_2$ requires C, 76·6; H, 9·6; N, 6·4%); *dihydrochloride*, rosettes or sheaves of colourless needles from water, softens at 120°, M.P. 180°.

n-*Decyl ether**. Separates as a jelly from light petroleum but on standing changes to felted needles, M.P. 111–113°, with some sintering at 107°, $[\alpha]_D^{15°}$ −120·8° (0·1 *N* $EtSO_3H$); *dihydrochloride*, granules made up of minute needles, M.P. 135–155°. (Found: loss on drying, 7·8; C, 65·8; H, 8·7; N, 5·6; Cl, 12·9%. Calc. for $2·5H_2O$, loss on drying, 7·9%. $C_{29}H_{44}O_2N_2.2HCl$ requires C, 66·2; H, 8·8; N, 5·3; Cl, 13·5%.)

*Undecyl ether**. Silky needles from a mixture of ether and light petroleum, sintering at 105°, M.P. 107–109°, $[\alpha]_D^{15°}$ −108·7° (0·1 *N* $EtSO_3H$). (Found: C, 77·1; H, 9·9; N, 6·1%. $C_{30}H_{46}O_2N_2$ requires C, 77·2; H, 9·9; N, 6·0%); *dihydrochloride*, minute cream-tinted rosettes of needles, M.P. 130–150°. (Found: Cl, 12·6%. B.2HCl requires Cl, 13·2%.)

As no alkyldihydrocupreidine ethers appear to have been subjected to antimalarial tests, four were made for this purpose. The ethyl ether had M.P. 199–201°, $[\alpha]_D$ +286·9° [cf. Heidelberger & Jacobs, 1919]. The other three already prepared by Ghosh & Chatterjee [1931; 1932] had the following constants: *iso*butyl, M.P. 178°, $[\alpha]_D^{20°}$ +271·6°; amyl, M.P. 161°, $[\alpha]_D^{21°}$ +253·1°; *iso*amyl, M.P. 169°, $[\alpha]_D^{20°}$ +246·8°.

Results of antimalarial tests.†

The anti-malarial tests were made by Roehl's method in canaries in the manner already described [Buttle *et al.* 1934]. This is now a standard method for

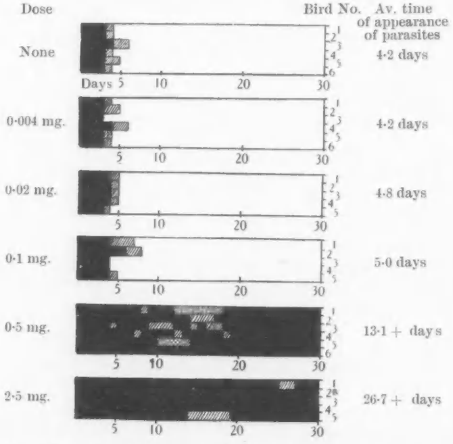


Fig. 1. Action of various doses of quinine on bird malaria: abscissae, time in days after infection; ordinates, serial number of individual birds.

The black areas represent the duration of time during which no parasites were seen for each individual bird; the cross-hatched areas the time during which parasites were seen in less than 1% of the corpuscles. When parasites appeared in 1% or more of the corpuscles the blood examination was discontinued. * One bird on 2·5 mg. was cured.

■ No parasites seen. ▨ Parasites in less than 1/100 R.B.C.
□ Parasites in more than 1/100 R.B.C.

the investigation of antimalarial drugs, and as quinine has been used throughout this investigation as a standard drug for comparison, it was considered advisable

† G. A. H. Buttle and J. W. Trevan are responsible for all the experiments on animals.

to determine the point at which antimalarial action can be detected for this alkaloid. The results are shown in Fig. 1, which shows the effects of the administration of a series of doses to groups of six canaries. Each dose was repeated daily for 6 days on and after the day of injection. The black columns represent the time in days from the infection to the appearance of an occasional parasite in the blood. The cross-hatched portion represents the further time elapsing till the parasites were present in 1% or more of the red cells. The average time of appearance of parasites is given against each dose. Where none appeared in 30 days of observation the "time of appearance" is taken as 30.

The curative effect of a given dose varies from experiment to experiment, not always in proportion to the intensity of the infection as judged by the time of appearance of parasites in the control birds. In this particular experiment the action of quinine is rather more pronounced than in the average.

A trace of effect first appears with 0·02 mg. of quinine; the mean time of appearance is 4·8 days instead of 4·2 for the control but the difference is probably not statistically significant. With 0·1 mg. doses the difference from the control is obvious, and doses of 0·5 mg. produce large increases in the time of appearance of parasites. Above this again the discrimination falls off. Between 0·1 and 0·5 mg. in this experiment doses in the ratio of 2 : 1 could probably be just distinguished. In other experiments the range of doses of quinine over which this degree of discrimination could be made would probably be slightly higher.

It would have been necessary to set up some such experiment as this with each drug, together with a corresponding group on various doses of quinine, to have established a quantitative ratio of activity for each drug. But as we were interested primarily in the order of activity and not in the establishment of the actual quantitative ratio to quinine, we adopted a simpler procedure, which involved the use of fewer birds. Constant doses of 2·5 mg. of quinine in one group of 4–6 birds and 2·5 mg. of the drug in another group were employed, and the average time of appearance of parasites was observed. These are recorded in columns 3 and 4 of Tables II, III and IV. In column 2 is given the average time of appearance of parasites in a control group of birds. Column 5 headed Quinine ratio is the ratio of the increase in the time of appearance of parasites in the drug-treated birds to the increase in time of appearance in the birds treated with quinine. It is obtained by dividing the difference between the figures in columns 3 and 2 by the difference between the figures in columns 4 and 2; where there is no difference between columns 3 and 2 the drugs are labelled Inactive. This means, if we consider the first experiment, that the activity is probably less than 1/25 of that of quinine. When the ratio is greater than 1 although the experiment indicates that the drug is more active than quinine we can give no sort of estimate of the ratio of activity except that the drugs with the longer retardation times relative to quinine are the more active. The Tables also include the number of birds cured as determined by re-infection experiments. This phenomenon is so erratic in appearance that very large numbers of birds would have to be used before any conclusions could be based on the proportion of cures obtained.

Table II. apo*Quinine and dihydrocupreine ethers*

Name of drug	Average time of appearance of parasites in peripheral blood: Controls days	Drug days	Quinine days	Quinine ratio	Birds cured: Drug	Quinine
*apo*Quinine (II: R = H)	5·5	18·4	18·5	0·98	—	1
apo*Quinine ethers* (II: R = Alkyl):						
Methyl (*β-iso*quinine)	5·0	16·4	21·5	0·69	—	—
Ethyl	5·5	21·0	18·6	1·18	1	1
Propyl	5·5	21·6	18·6	1·23	1	1
*iso*Propyl	5·3	10·0	14·5	0·51	—	1
n-Butyl	4·2	22·2	20·75	1·1	—	1
n-Amyl	4·2	28·6	20·75	1·47	1	1
*iso*Amyl	5·3	17·0	14·5	1·27	—	1
n-Hexyl	4·3	21·0	14·0	1·72	—	—
n-Heptyl	4·3	16·0	14·0	1·21	—	—
n-Octyl	4·3	22·0	14·0	1·82 } Av.	2	—
	4·4	19·5	15·4	1·36 } 1·6	—	—
sec-Octyl	4·4	20·4	15·4	1·44	1	—
	5·0	18·0	25·5	0·65 } Av.	—	—
	5·0	20·8	26·2	0·745 } 0·98	1	—
	7·9	29·0	27·5	1·08	3	3
n-Nonyl	4·2	20·3	20·75	0·958 } 0·98	—	1
	4·0	24·0	24·0	1·0	—	—
n-Decyl	4·2	21·8	20·75	1·32 } 1·18	—	—
	4·0	25·0	24·0	1·05	—	—
n-Undecyl	4·0	19·0	24·0	0·75	—	—
Cetyl	7·7	17·0	27·5	0·47 } Av.	1	3
	5·0	19·3	26·2	0·69 } 0·58	—	—
*isoapo*Quinine (II: R = H)	4·0	12·0	13·0	0·9	—	—
Methyl ether (*α-iso*quinine)	5·0	18·1	26·2	0·62	—	—
Dihydrocupreine (VI: R = H)	5·0	23·0	21·5	1·109 } Av.	—	—
	4·0	12·0	14·7	0·75 } 0·92	—	—
Dihydrocupreine ethers (VI: R = Alkyl):						
Methyl (dihydroquinine)	7·2	26·2	21·0	1·35	—	1
Methyl (*epi*-C^9-dihydro-quinine)	4·0	21·0	21·3	1·0	—	1
Ethyl	5·5	19·3	18·6	1·05	—	1
Propyl	5·3	19·0	14·5	1·49	—	1
*iso*Propyl	5·5	23·3	18·6	1·37	2	1
n-Butyl	5·3	22·5	14·5	1·87	1	1
n-Amyl	5·5	19·3	18·6	1·33	2	1
*iso*Amyl	5·3	22·0	14·5	1·83	—	1
n-Hexyl	4·3	16·5	14·0	1·26 } Av.	1	—
	4·3	21·0	14·0	1·73 } 1·5	—	—
n-Heptyl	4·0	>30·0	24·0	>1·3 } Av.	—	—
	4·3	22·0	13·0	2·04 } 1·70	—	—
n-Octyl	4·3	19·4	14·0	1·56 } Av.	—	—
	4·0	23·0	15·4	1·67 } 1·43	—	—
	4·0	20·4	19·2	1·08	—	—
sec-Octyl	4·3	24·5	16·0	1·53	1	—
n-Nonyl	4·3	18·0	16·0	1·12	1	—
n-Decyl	4·3	25·7	16·0	1·6	2	—
n-Undecyl	4·3	20·0	16·0	1·25	—	—

Table III. apo*Quinidine and dihydrocupreidine ethers*

Name of drug	Average time of appearance of parasites in peripheral blood: Controls days	Drug days	Quinine days	Quinine ratio	Birds cured: Drug	Quinine
*apo*Quinidine (II: R = H)	5·0	11·6	21·5	0·4	—	—
Methyl ether	4·0	13·0	13·0	1·0	—	—
*neoapo*Quinidine (II: R = H)	7·7	16·6	27·5	0·465	—	3
ψ-Quinidine (IV: R = Me)	3·0	17·0	20·6	0·79	—	—
*isoapo*Quinidine (V: R = H)	5·5	7·0	18·6	0·11	—	1
isoapo*Quinidine ethers* (V: R = Alkyl):						
Methyl (*β-iso*quinidine)	5·0	5·6	21·5	0·1	—	—
Ethyl	4·3	7·0	14·0	0·28 } Av.	—	—
	4·0	6·0	13·0	0·22 } 0·25	—	—
n-Amyl	4·0	6·0	13·0	0·22	—	—
*iso*Amyl, *n*-hexyl, *n*-heptyl, *n*-octyl	4·0–4·3	4·0–5·0	13·0–14·0	Inactive	—	—
*α-iso*Quinidine (V: R = Me)	7·7	9·0	27·5	0·065	—	3
*γ-iso*Quinidine (V: R = Me)	4·0	<6·0	21·3	Inactive	—	1
Dihydrocupreidine (VI: R = H)	5·0	16·2	21·5	0·68	—	—
Dihydrocupreidine ethers (VI: R = Alkyl):						
Methyl (dihydroquinidine)	3·0	17·4	20·6	0·81	—	—
Ethyl	5·0	24·0	25·0	0·95 } Av.	1	1
	5·0	18·0	25·0	0·65 } 0·98	—	—
	5·0	27·2	21·5	1·35 }	1	—
*iso*Butyl	4·0	20·0	24·0	0·8	—	—
n-Amyl	4·0	12·0	24·0	0·4	—	—
*iso*Amyl	4·0	17·0	24·0	0·65	—	—

Table IV. *Various quinine and quinidine transformation products*

Name of drug	Average time of appearance of parasites in peripheral blood: Controls days	Drug days	Quinine days	Quinine ratio	Birds cured: Drug	Quinine
Hydroxy-bases (III: R = H or Me):						
α-Hydroxydihydro*apo*quinine	5·3	6·0	14·5	0·076	—	1
Methyl ether (hydroxydihydroquinine)	5·0	16·4	26·2	0·542	—	—
β-Hydroxydihydro*apo*quinine	5·0	5·6	21·5	0·036	—	—
epi-*Bases*:						
epi-C⁹-Quinine (I: R = Me)	4·5	6·5	24·7	0·099 } Av.	—	—
	5·0	<7·0	25·0	0·1 } 0·1	—	—
epi-C⁹-Quinidine (I: R = Me)	4·5	6·2	24·7	0·084 } Av.	—	—
	3·0	<7·0	25·0	0·181 } 0·13	—	—
epi-C⁹-Dihydroquinine (VI: R = Me)	4·0	21·0	21·3	1·0	—	1
epi-(C⁹-Dihydroquinidine (VI: R = Me)	4·0	16·0	20·7	0·72	—	—
Niquine (VII, Léger)	4·2	23·7	26·2	0·86	—	1
Dihydroniquine	3·0	14·2	20·6	0·63	—	—
Niquidine	4·0	22·0	16·4	1·45	1	—
*iso*Niquidine	4·0	17·0	16·4	1·05	—	—
Dihydroniquidine	4·0	22·4	21·3	1·06	—	—
Quinine dihydrochloride zinci-chloride, $B.2HCl.ZnCl_2$	7·7	28·5	27·5	1·05 } Av.	4	3
	4·0	17·2	21·3	0·81 } 0·93	—	1

Discussion of biological results

It is remarkable that whilst epimerization at C^9 reduces the activity of quinine from 1 to 0·1 in *epi*-C^9-quinine and that of quinidine from 0·5 to 0·1 in *epi*-C^9-quinidine [cf. Dirscherl & Thron, 1935] and that whilst dihydroquinine has a quinine ratio of 1·35, *epi*-C^3-dihydroquinine has the same activity as quinine, there is no obvious relationship traceable between optical activity and antimalarial action in the cinchona alkaloids; thus while it is true that the *l*-bases, quinine and cinchonidine, are more active than the *d*-bases, quinidine and cinchonine [Buttle *et al.* 1934], *apo*quinidine methyl ether (+334·2°) is more active (quinine ratio 1·0) than either of its *l*-isomerides α- or β-*iso*quinine (quinine ratios 0·62 and 0·69 respectively). It is also of interest in this connexion to note that, whilst conversion of the phenolic *l*-bases *apo*quinine and *isoapo*quinine into their methyl ethers β- and α-*iso*quinines results in a reduction of antimalarial action, the converse is the case when the *d*-phenolic base, *apo*quinidine, is methylated.

It is not surprising that the ether oxide type of structural isomeride (V: R=Me) represented by α-, β- and γ-*iso*quinidines should prove to be almost inactive, since it is well known that any modification of the structure of the —CHOH— group connecting the two halves of the cinchona alkaloids reduces antimalarial activity.

The most interesting results are to be found in the homologous series of *apo*quinine and dihydrocupreine ethers, as will be seen from Table V, in which

Table V

Quinine ratio		*apo*Quinine ethers	Name of ether	Dihydrocupreine ethers	Quinine ratio	
Even	Odd	Formula		Formula	Odd	Even
0·69	—	$C_{20}H_{24}O_2N_2$	Methyl	$C_{20}H_{26}O_2N_2$	—	1·35
—	1·18	$C_{21}H_{26}O_2N_2$	Ethyl	$C_{21}H_{28}O_2N_2$	1·05	—
1·23	—	$C_{22}H_{28}O_2N_2$	*n*-Propyl	$C_{22}H_{30}O_2N_2$	—	1·49
—	1·10	$C_{23}H_{30}O_2N_2$	*n*-Butyl	$C_{23}H_{32}O_2N_2$	1·87	—
1·47	—	$C_{24}H_{32}O_2N_2$	*n*-Amyl	$C_{24}H_{34}O_2N_2$	—	1·33
—	1·72	$C_{25}H_{34}O_2N_2$	*n*-Hexyl	$C_{25}H_{36}O_2N_2$	1·50	—
1·21	—	$C_{26}H_{36}O_2N_2$	*n*-Heptyl	$C_{26}H_{38}O_2N_2$	—	1·70
—	1·6	$C_{27}H_{38}O_2N_2$	*n*-Octyl	$C_{27}H_{40}O_2N_2$	1·43	—
0·98	—	$C_{28}H_{40}O_2N_2$	*n*-Nonyl	$C_{28}H_{42}O_2N_2$	—	1·12
—	1·18	$C_{29}H_{42}O_2N_2$	*n*-Decyl	$C_{29}H_{44}O_2N_2$	1·6	—
0·75	—	$C_{30}H_{44}O_2N_2$	*n*-Undecyl	$C_{30}H_{46}O_2N_2$	—	1·25
—	0·58	$C_{35}H_{54}O_2N_2$	Cetyl	Not prepared	—	—

the quinine ratios are assembled. If each homologous series is considered as a whole there appears to be no regularity in rise or fall of the quinine ratio, but if each series is divided into members containing odd and even numbers of carbon atoms, it will be seen that, with some anomalies, there is a definite tendency in the four series so produced for the quinine ratio to rise to a maximum and then fall as the series is ascended.

This is not the first instance of this curious alternation in the parasiticidal activity of members of a homologous series; it was first noticed by Magidson [Magidson & Strukow, 1933; Magidson *et al.* 1935] in testing the antimalarial activity in infected siskins of a series of 6-methoxyquinolines substituted in position 8 by the chain —NH—$[CH_2]_n$—NEt_2. These authors found the following chemotherapeutic indices (*i*) for different values of *n*.

n	2	3	4	5	6	7	8	9	10	11
i	—	26	—	25	—	34	—	40	—	5
i	6	—	11	—	13	—	—	—	—	—

This alternation was confirmed by Bovet *et al.* [1934], who tested the same series on Java sparrows, but in this instance it was observed that as the series was ascended the drug acquired schizonticidal action in addition to its original gametocidal character.

Alternation is not, however, always shown in such tests with homologous series. Magidson & Strukow [1933] found that when the chain in position 8 was kept constant at —NH—$[CH_2]_2$—NEt_2 and the group in 6 varied from HO— to $C_5H_{11}O$—, there was a continuous but irregular fall in the chemotherapeutic index from 13 to 0. Similarly in a short series of substituted acridines with methoxyl at position 7, chlorine at position 3 and at position 5 the chain —NH—$[CH_2]_n$—NEt_2, where $n=2$ to 6, the chemotherapeutic index began at 8, rose to 20 at $n=4$ and fell to 6 and 5 at $n=5$ and 6 respectively [Magidson & Grigorovski, 1936]. These series are very short and the rise or fall is much more abrupt than in the instances in which the alternating effect is shown, but as they stand there is no reason to suppose that an alternating effect has been obscured by the particular experimental conditions. Comparatively few investigations have been made of the change in action *in vivo* exhibited by a homologous series of parasiticidal drugs, though the results of numerous *in vitro* investigations of this kind are available. In the latter the results generally show a rise to a maximum followed by a fall as the series is ascended and may be explained as resulting from the action of two opposing factors, e.g. parasiticidal action conditioned by the solubility or some other physical property of the drug used. It has been generally assumed that results obtained *in vivo* would show the same tendency, but the results of the Russian workers, and those now recorded, seem to indicate that when such investigations are conducted *in vivo* some new factor may come into play which leads to the alternation in the effect of length of chain on activity.

The short series of dihydrocupreidine ethers (Table III) is mainly of interest as showing that in the dextrorotatory bases a rise in activity also occurs as the side-chain is increased in length.

The results given by the hydroxy *apo*-bases (Table IV) are perhaps to be expected since the presence of a phenolic hydroxyl group in the quinoline nucleus, and an additional hydroxyl group attached to carbon atom 10, reduces the basic character, which seems to be essential for antimalarial action and it is noticeable that the activity is largely increased by methylation of the phenolic hydroxyl group.

The activities of niquine and the two niquidines are surprising, niquidine (quinine ratio 1·45) being much the most active of the dextrorotatory cinchona alkaloids yet known. The zincichlorides have proved so useful as a means of isolating transformation products of the cinchona alkaloids, being sometimes the only crystallizable derivatives obtainable, that it seemed desirable to ascertain whether in case of necessity they could be used for antimalarial tests. In the single case tried, quinine dihydrochloride zincichloride (Table IV) the complex salt seemed to behave like any other salt of quinine.

It is not yet possible to draw any precise general conclusions as to the mode of action of the cinchona alkaloids as antimalarial drugs. It seems to be clearly established that they act directly on the parasites and that any modification in structure which results in the formation of a weaker base, as in the conversion of quinine into quitenine, reduces or extinguishes their activity. That inactivity ensues from even a spatial distribution change as well as from small structural changes in the connecting —CHOH— group seems to imply that the essential condition for activity is a close "fit" between the drug and the protein molecules

of the parasite, and that view seems to be supported by the alternation in activity shown by successive members of homologous series.

The authors desire to place on record their thanks for a gift of *epi*-C^9-quinine and *epi*-C^9-quinidine from Dr Thron, who in conjunction with Dr Dirscherl [1935] found these interesting substances inactive in avian malaria. They are also indebted to Messrs L. E. Barnett, R. Maidstone and A. W. Stokes for assistance in the preparatory work and to Messrs A. Bennett and H. C. Clarke for micro-analyses.

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XI. STUDIES ON BAYER 205 (GERMANIN) AND ANTRYPOL

I. THE DETERMINATION OF SMALL AMOUNTS OF BAYER 205 (AND ANTRYPOL)

II. THE PERSISTENCE OF BAYER 205 IN THE BLOOD STREAM AFTER INJECTION INTO ANIMALS

By WILLIAM GARDINER DANGERFIELD, WILLIAM ERIC GAUNT and ARTHUR WORMALL

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(*Received 30 November 1937*)

Bayer 205 and Antrypol, besides having very high curative effects in trypanosomiasis, have been shown to possess marked prophylactic or preventive properties as far as this disease is concerned. Although the *in vivo* trypanocidal action of these drugs has not yet been fully explained, it seems probable that their prolonged prophylactic action is due to the fact that they are retained in the blood and probably in the tissues for some considerable time after injection. It seems desirable, therefore, that more information should be obtained about the "metabolism" of these drugs in the body. At a conference on Tsetse and Trypanosomiasis (Animal and Human) Research held at Entebbe a few years ago [Report, 1934] the view was expressed "that it was important that the question of the rapidity of excretion of Bayer 205 from the body should be investigated" and "it was recommended that the Medical Research Council should be approached with a request to work on this problem". The Medical Research Council having taken up the matter accordingly, it was at their request that the experiments described in this paper were made.

The first essential was an accurate method for the determination of Bayer 205 in plasma, and if possible, in other body fluids, and after much search, the method described in the first part of this paper was devised. This method gives satisfactory results with plasma, but considerable difficulties were encountered when attempts were made to apply it to urine. Various modifications for the urine determinations have been tried and although some are quite promising, it is not possible as yet to place any great reliance on the results obtained. The present report will therefore be confined to the problem of the retention of Bayer 205, and the mode of elimination will be left to a future date. These early investigations have been made on small animals (rabbits and dogs), but similar experiments are being carried out with man as the experimental animal. At the same time efforts are being made to determine the minimum plasma Bayer 205 level which will prevent infection when various strains of trypanosomes are injected into small animals.

of the parasite, and that view seems to be supported by the alternation in activity shown by successive members of homologous series.

The authors desire to place on record their thanks for a gift of *epi*-C^9-quinine and *epi*-C^9-quinidine from Dr Thron, who in conjunction with Dr Dirscherl [1935] found these interesting substances inactive in avian malaria. They are also indebted to Messrs L. E. Barnett, R. Maidstone and A. W. Stokes for assistance in the preparatory work and to Messrs A. Bennett and H. C. Clarke for micro-analyses.

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The first essential was an accurate method for the determination of Bayer 205 in plasma, and if possible, in other body fluids, and after much search, the method described in the first part of this paper was devised. This method gives satisfactory results with plasma, but considerable difficulties were encountered when attempts were made to apply it to urine. Various modifications for the urine determinations have been tried and although some are quite promising, it is not possible as yet to place any great reliance on the results obtained. The present report will therefore be confined to the problem of the retention of Bayer 205, and the mode of elimination will be left to a future date. These early investigations have been made on small animals (rabbits and dogs), but similar experiments are being carried out with man as the experimental animal. At the same time efforts are being made to determine the minimum plasma Bayer 205 level which will prevent infection when various strains of trypanosomes are injected into small animals.

I. THE DETERMINATION OF SMALL AMOUNTS OF BAYER 205 (AND ANTRYPOL)

Two methods for the determination of Bayer 205 in body fluids have been described. Steppuhn and Utkina-Ljubowzewa [1924] coupled the products obtained by alkaline hydrolysis of Bayer 205 with *p*-diazobenzenesulphonic acid in acetic acid solution, and compared the orange colour obtained with standards. This method has been criticized by Lang [1931] on the grounds that loss of the material to be determined occurs during alkaline hydrolysis and further that the diazo-compound couples with substances normally present in the blood to give coloured compounds. The present authors have confirmed these findings of Lang and have additional criticisms of the method to offer. When determinations are made on blood serum or plasma, protein precipitates are produced which carry down a certain amount of the orange colour which is ultimately produced. In the case of determinations on urine, it has been found that normal urines invariably give a very marked coloration when the diazo-compound is added. In view of these facts it is concluded that this method is not suitable for the detection or determination of Bayer 205 in body fluids.

Attention was then turned to the method of Lang [1931] in which the aromatic amines liberated by the acid hydrolysis of Bayer 205 (*m*-aminobenzoic acid, *m*-amino-*p*-toluic acid and α-naphthylamine-4:6:8-trisulphonic acid) are diazotized and coupled, in acetic acid solution, with α-naphthol. The orange-yellow colour produced is estimated colorimetrically by comparison with a similarly treated standard solution of Bayer 205. Although the method is distinctly better than that of Steppuhn and Utkina-Ljubowzewa, it is not completely satisfactory. The acid hydrolysis employed in this technique leads to the production of brown or black pigments from urine and from blood plasma or serum. The pigments are partially removed by treatment with kaolin and filtration, but the "urine" filtrates have a yellow colour which is similar to that ultimately produced when the α-naphthol is coupled with the diazotized hydrolysed Bayer 205. The filtrates from plasma and serum also contain pigments which are not completely removed by kaolin and which interfere with the subsequent matching of the orange-yellow colours.

Attempts were made to overcome these difficulties by the use of coupling agents other than α-naphthol in the hope of producing deeper colours such as reds or blues. Tests were made with a large number of *N*-substituted naphthylamines and other compounds used for coupling with diazo-compounds, and ultimately it was found that methyl-α-naphthylamine gave a magenta colour when coupled in acetic acid solution with the diazotized products of the acid hydrolysis of Bayer 205. Subsequent experiments showed that the coupling was quantitative, and further, that the slight brownish yellow colour of hydrolysed and kaolin-treated plasma interfered to a very slight extent only with the matching of the colours. The method for the estimation of small amounts of Bayer 205 and of Antrypol, described in this paper is, therefore, a modification of the Lang technique. The modifications have been introduced in order to obtain the optimum conditions for the production and stabilization of the magenta colour obtained when methyl-α-naphthylamine is employed as coupling agent.

Description of the method

The principle of the method consists in hydrolysing Bayer 205 (or Antrypol) with conc. HCl; the aromatic amines produced are diazotized, and after removal of excess of HCl with sodium acetate the diazo-compounds are coupled with monomethyl-α-naphthylamine. The magenta colour produced is compared with that obtained from a similarly treated standard solution of Bayer 205 or Antrypol. The methyl-α-naphthylamine was prepared according to Fischer [1895]. It can also be purchased from Messrs May and Baker, Ltd., Dagenham.

The following reagents are required:

(1) Concentrated HCl.
(2) Kaolin (Society of Leather Trades Chemists' specification).
(3) 0·5% sodium nitrite.
(4) Saturated urea solution.
(5) 30% sodium acetate.
(6) 0·5 ml. methyl-α-naphthylamine (or 0·2 g. of the hydrochloride) in 100 ml. of 50% acetic acid (% by volume).

To 1–3 ml. of the oxalated plasma in a 10 ml. graduated tube, are added 3 ml. of conc. HCl. A glass bulb is placed in the top of the tube, which is then heated in the boiling water bath for 6 hr. The cooled brown or brownish black mixture is diluted with distilled water to 10 ml., treated with approximately 3 g. of previously heated kaolin, shaken well and filtered. 2 ml. of the filtrate are treated with 1 drop of 0·5% $NaNO_2$, followed after 1 min. by 2 drops of saturated urea solution, the mixture being well shaken after each addition. After 2 min., 3 ml. of 30% sodium acetate solution are added, followed immediately by 1 ml. of the methyl-α-naphthylamine solution. The colour develops rapidly and after 10–15 min. is matched colorimetrically against a standard.

The standard solutions are prepared as follows. A solution of Bayer 205 (e.g. 100 mg./100 ml.) is treated with an equal volume of conc. HCl and heated for 6 hr. in the boiling water bath. From this solution, which appears to be stable for a considerable time, various standards are made by dilution with 3*N* HCl, the most useful being those containing 0·25, 0·5, 0·75, 1·0, 1·5, 2·0 and 3·0 mg. Bayer 205 per 100 ml. 2 ml. of each standard are diazotized, treated with urea and coupled with methyl-α-naphthylamine as described above. If the solution under examination gives a deeper colour than that produced by the 3 mg./100 ml. standard, less of the filtrate is used, 3*N* HCl being added to give a volume of 2 ml. before diazotization. Solutions giving a less intense colour than that produced by the 0·5 mg./100 ml. standard, or solutions containing so much yellow colour that matching in the colorimeter is difficult, are matched in a comparator. The solution used for compensation in these cases consists of the filtrate treated with $NaNO_2$, urea, sodium acetate and 1 ml. of 50% acetic acid.

The concentration of Bayer 205 in the biological fluid (in mg. per 100 ml.) is given by the following formula:

$$\frac{10}{v_1} \times \frac{2}{v_2} \times \frac{a}{b} \times S,$$

where v_1 = volume of fluid hydrolysed (plasma, serum etc.); v_2 = volume of filtrate diazotized; a = colorimeter reading of standard; b = colorimeter reading of solution; S = concentration of standard (mg. Bayer 205 per 100 ml.).

The following points have been found to be important.

(1) The correct balance between HCl and sodium acetate must be main-

tained. Any dilution of the "kaolin-filtrate" should be made with 3*N* HCl, and 2 ml. of this diluted solution used for the preparation of the red colour.

(2) No excess of nitrous acid should remain in the solution after the treatment with urea. The contents of the tube should be well shaken to make certain that no nitrous acid remains on the side of the tube.

(3) It is important that the prescribed time should be allowed for diazotization and for the destruction of excess nitrous acid. Most satisfactory results are obtained when these processes are accurately timed.

(4) The methylnaphthylamine reagent should be added immediately after the sodium acetate.

(5) Normal sera usually give a small blank value, presumably due to amines present in the plasma or produced by the hydrolysis. This blank value for rabbits appears to vary slightly from time to time, but the cause of this variation has not yet been established. In these investigations determinations have been made on control animals and the values given in this paper have, where necessary, been corrected for the small blank (usually equivalent to 0·8 or 0·9 mg./100 ml. of Bayer 205).

When attention is paid to these points the method is relatively simple and gives satisfactory results when duplicates are compared and when determinations are made on sera or plasmas containing known amounts of Bayer 205 (cf. Table I).

Table I. *Determination of Bayer 205 (and Antrypol) in water, plasma and serum*

Medium	Amount of drug added mg./100 ml.	Amount recovered mg./100 ml.
Water	10·0 Bayer 205	9·9
	5·0 ,,	5·1
	2·0 ,,	2·0
	10·0 Antrypol	10·0
	5·0 ,,	5·0
Plasma (rabbit)	5·0 Bayer 205	5·1
	2·0 ,,	2·2
Serum (horse)	11·4 Antrypol	11·3
	5·7 ,,	5·6
	2·3 ,,	2·4
	5·7 Bayer 205	5·9
	2·3 ,,	2·4

Methyl-α-naphthylamine undergoes some decomposition, especially when exposed to light, and becomes red-brown. This colour is not usually sufficient to necessitate correction of the results, but in more recent work it has been found desirable to purify the reagent by conversion into the hydrochloride. Experiments so far carried out show that the solid hydrochloride undergoes no apparent decomposition when kept in the dark for several weeks. It is also more economical to use, for the 0·5% solution of the base can be replaced by a solution containing 0·2 g. of the hydrochloride in 100 ml. of 50% acetic acid (by volume). Where neither the base nor the hydrochloride is available, recrystallized α-naphthylamine can be used but the colour with this reagent is not quite as bright a red, does not develop as quickly, and is more difficult to match with the standards than is the colour with methyl-α-naphthylamine.

Very recently efforts have been made (in collaboration with Dr F. Hawking) to utilize a Lovibond comparator for this determination, and coloured discs are

being made by "The Tintometer Ltd." When this object has been achieved it is hoped that determinations of Bayer 205 will be possible in small field laboratories in tropical countries where a colorimeter is not available.

Discussion

(*a*) *The hydrolysis of Bayer 205 and Antrypol.* In agreement with Lang [1931] it has been found that if a mixture of equal volumes of plasma and conc. HCl is heated in a boiling water bath, hydrolysis of any Bayer 205 present is complete in 5–6 hr. (cf. Table II). Heating for 3 or 4 hr. longer causes very little loss of the amines produced by the hydrolysis. Almost identical results, both as regards the amount present in solution and the rate of hydrolysis by HCl, have been obtained with Antrypol (B.D.H.). In all the experiments carried out with this colorimetric determination, Antrypol has behaved exactly like Bayer 205, and for these determinations standards prepared from either of the hydrolysed drugs can be used. In view of the considerable difference in the cost of these two preparations it is proposed to carry out most of the later experiments with Antrypol.

Table II. *Rates of hydrolysis of Bayer 205 and Antrypol. % of Bayer 205 or Antrypol hydrolysed*

Bayer 205	57·1	79·1	90·7	95·5	98·1	100·0	100·0	99·1
Antrypol	54·1	79·5	89·9	95·1	99·3	99·7	100·0	98·4
Time (hr.)	1	2	3	4	5	6	8	10

Experimental details

Solutions of Bayer 205 and Antrypol containing 10 mg./100 ml. were treated with an equal volume of conc. HCl. Twenty 6 ml. samples of each solution were placed in a boiling water bath and two tubes of each set were removed after various intervals. The amount of hydrolysed Bayer 205 in each tube was determined and the average figure for each pair of duplicates was used for the calculation of the percentages given in the table. Solutions of hydrolysed Bayer 205 were used as standards for these determinations.

(*b*) *Decoloration.* Lang [1931] states that kaolin removes the dark-coloured pigments present in hydrolysed plasma without loss of the products of hydrolysis of Bayer 205. This observation has received general confirmation in the present investigations, but it must be recorded that with solutions containing small amounts of Bayer 205 (less than 2 mg./100 ml.) treatment with kaolin results in a small but detectable loss (equivalent to about 0·15 mg./100 ml. of Bayer 205). Where necessary, allowance should be made for this adsorption. No adjustment is needed with plasma determinations, since there is a corresponding loss in the blank.

Decoloration with kaolin is not complete, particularly in the case of urine estimations, and search has been made for some more satisfactory method. Other adsorbents have been found to be unsatisfactory, for removal of the pigments is usually accompanied by a loss of Bayer 205. Heated kaolin is, however, a little more effective than the unheated material, and in all the later experiments the kaolin has been heated moderately strongly in a boiling tube for about 1 min. before addition to the diluted hydrolysed plasma.

(*c*) *Diazotization and coupling.* In the early stages of the experiments it was found that the colour produced by coupling the diazotized products of acid hydrolysis of Bayer 205 with methyl-α-naphthylamine was slowly destroyed, and this instability was traced to the presence of an excess of nitrous acid. The

addition of urea before neutralization with sodium acetate removes this excess of nitrous acid and the colour then remains constant for several hours.

It was also found that whilst the presence of excess of HCl diminishes both the depth of the colour and the rapidity with which it is produced, excess of sodium acetate, which aids both these factors, makes the solution turbid owing to the precipitation of methyl-α-naphthylamine. It is necessary, therefore, to control the amounts of HCl and sodium acetate. Conditions were ultimately chosen which gave the most rapid production of a maximal colour which would remain clear for at least 2 hr. Thus the 2 ml. sample used for analysis must be approximately 3*N* in HCl and the sodium acetate added after diazotization must be 30%. If turbidity is produced in individual solutions, they must be cleared by centrifuging, but this is rarely necessary.

It is also important that the methyl-α-naphthylamine solution should be added immediately after the sodium acetate. Any appreciable delay at this stage causes loss of the diazo-compounds, probably owing to their coupling with compounds present in the hydrolysed plasma. As the solution of methyl-α-naphthylamine becomes darker on standing, it is necessary to make up this reagent every 3–4 days.

(*d*) *Accuracy of the method.* The method has proved satisfactory for the determination of small amounts of Bayer 205 and Antrypol, and it gives fairly accurate results with even the smallest amounts of these drugs which have been shown to exercise a definite trypanocidal effect. With aqueous solutions as little as 0·2 mg./100 ml. of Bayer 205 can readily be detected, and accurate determinations made when the concentration exceeds 0·6 mg./100 ml. In the case of plasma, where an allowance for the blank value must be made, it is possible to detect 0·4 mg./100 ml. and to determine with sufficient accuracy amounts greater than 0·6 mg./100 ml. When the concentration in the plasma exceeds 2 mg./100 ml., the error is not greater than that normally associated with the matching of colours in a colorimeter (cf. Table I).

Up to the present it has not been found possible to make accurate determinations of Bayer 205 in urine, for the blank values obtained for normal urines, with this or any similar method, are considerable and are not sufficiently constant to warrant any conclusion being reached as to the amount of Bayer 205 present. Amines and possibly other diazotizable substances are normally found in the urine to such an extent and with such wide variation in the amounts (equivalent to 2–5 mg./100 ml. of Bayer 205), that the method described here, and still more those of Steppuhn and Utkina-Ljubowzewa [1924] and Lang [1931], can only be used to give an approximate value for Bayer 205 in urine. Attempts have been made to remove the diazotizable substances from the urine before hydrolysis, but the results are not yet sufficiently satisfactory to justify the publication of this modification.

It should be pointed out that this method, and any other method involving the same principles, can only give a figure for the amount of Bayer 205 plus related substances present in the plasma. It does not appear likely that there will be any appreciable amount of simple amines in the blood plasma following the injection of Bayer 205, but there is still the possibility that this drug is partially degraded or converted into other derivatives which remain in the blood stream. These Bayer 205 derivatives would probably be hydrolysed by HCl to give amines which would give red colours when diazotized and coupled with methyl-α-naphthylamine. There is evidence, however, that after its injection part of the Bayer 205 is retained in the body in the form of the unchanged compound. In the experiments recorded in the second part of this paper the

assumption is made that the results obtained are a measure of the amount of unaltered Bayer 205 in the plasma, but further work is proceeding to determine, if possible, whether there is any degradation of this drug in the animal body.

II. THE PERSISTENCE OF BAYER 205 IN THE BLOOD STREAM AFTER INJECTION INTO ANIMALS

It has long been recognized that the injection of Bayer 205 into animals infected with trypanosomes not only causes disappearance of the trypanosomes from the blood but also confers on the animals an immunity towards this type of infection. This immunity lasts for a varying length of time, depending on the amount of drug injected, the nature of the animal and the infection, and possibly on a variety of other factors. The prophylactic action of Bayer 205 has been demonstrated with various small animals (the rat, guinea-pig and monkey) and with man, and the immunity produced is effective against the bites of infected tsetse flies or the injection of trypanosomes into the animal. (For a survey of the literature on this subject the review of Findlay [1930], and the papers of Mayer [1928], Browning & Gulbransen [1934] and Duke [1934] should be consulted.)

The fact that a single injection of the drug into a monkey, an animal which is very sensitive to *T. rhodesiense* and *T. brucei*, will give protection for at least one month against infection from the bite of tsetse infected with these trypanosomes, "places Bayer 205 in a class by itself as a prophylactic of unique properties and immense possibilities" [Duke 1934]. Certain drugs can be used to give protection against other diseases, but there appear to be few instances where the prophylactic effect lasts for more than a few days. One exception to this general rule is the remarkable protection, against *T. brucei* infection, conferred by one subcutaneous injection of "Styryl-245" [Browning & Gulbransen, 1934]. This protection may last up to almost one year, and the prolonged effect is attributed to the formation of a local deposit from which the active substance passes very slowly into the blood stream. A similar explanation could not, of course, be offered to explain the long protective action of Bayer 205. Little is known about the length of time that this drug remains in the body after injection, or about the chemical explanation for its retention. The trypanocidal effect of Bayer 205 may be due to a direct action on the trypanosomes or an indirect mechanism, but whatever the reason, it is important that more should be known about the "metabolism" of this drug in the animal body.

Experimental

The determinations of Bayer 205 or Antrypol in the plasma of rabbits and dogs were carried out by the method described in Part I of this paper. Samples of blood (6–10 ml.), removed from the marginal vein of the ear, were oxalated and then centrifuged almost immediately. Determinations were occasionally made on duplicate samples of plasma, but generally only single samples were used. In these latter cases duplicate determinations were made on the filtrate produced after hydrolysis and kaolin treatment of the plasma. For injection, the Bayer 205 and Antrypol were dissolved in 0·9% NaCl and the solutions sterilized by boiling. The injections were all made intravenously.

Table III. *Amount of Bayer 205 (mg./100 ml.) in the blood of rabbits after a single injection of 0·1 g. per kg.*

Rabbit No. 468	201	142	—	73	48	33	30	21	14·5
Rabbit No. 449	204	—	86	—	51	32	25	18	11
Time after injection	5 min.	30 min.	1 hr.	3 hr.	6 hr.	24 hr.	30 hr.	48 hr.	4 days

Rabbit No. 468	13·5	10·5	rabbit died				
Rabbit No. 449	10	6·2	2·6	2·5	2·0	1·2	0·5
Time after injection	6 days	11 days	6 wks.	14 wks.	19 wks.	23 wks.	30 wks.

Table IV. *Bayer 205 in the plasma of rabbits following injection of the drug (in mg. per 100 ml.)*

	Group A. One large injection				Group B. Five small injections					
Time after injection	No. 486	No. 483	No. 484	Av.	Time after first injection	Time after previous injection	No. 480	No. 481	No. 482	Av.
→					→					
2½ hr.	88	94	85	89	2½ hr.	2½ hr.	29·3	33	29·0	30·4
4 days	24·7	20·6	25·7	23·7	4 days	4 days	2·7	5·2	3·0	3·6
					→					
8 ,,	14·0	14·5	15·7	14·7	8 days	20 hr.	9·0	16·0	11·0	12·0
14 ,,	9·0	11·0	12·0	10·7	14 days	7 days	3·0	7·0	3·8	4·6
					→					
21 ,,	5·0	7·3	7·0	6·4	21 ,,	7 ,,	7·1	6·7	5·7	6·5
					→					
32 ,,	4·0	6·3	5·3	5·2	32 ,,	11 ,,	6·6	6·5	5·2	6·1
					→					
39 ,,	3·1	4·4	3·7	3·7	39 ,,	7 ,,	8·4	9·4	8·4	8·7
53 ,,	3·2	4·2	3·5	3·6	53 ,,	21 ,,	6·4	6·4	6·6	6·5
67 ,,	2·0	3·0	2·0	2·3	67 ,,	35 ,,	4·5	3·1	4·5	4·0
90 ,,	1·7	1·8	1·6	1·7	90 ,,	58 ,,	2·4	1·4	2·9	2·2
17 weeks	1·0	1·5	1·1	1·2	17 weeks	12 weeks	1·8	1·5	2·1	1·8
25 ,,	0·8	1·1	1·0	1·0	25 ,,	20 ,,	1·1	1·0	1·2	1·1

Group A received one injection of 0·14 g. Bayer 205 per kg.
Group B received five injections of 0·028 g. Bayer 205 per kg.
The arrows in the above table indicate when the injections were made.

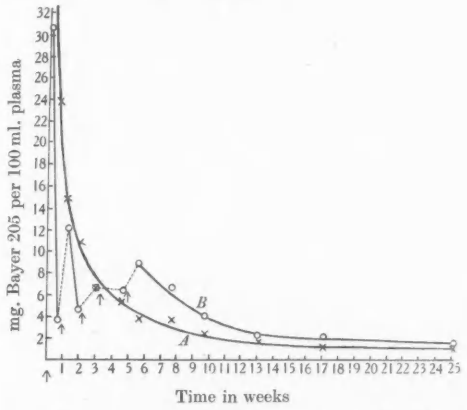

Fig. 1. Bayer 205 in the plasma of rabbits following the injection of the drug.
× — × Group A (one injection). o—o Group B (five injections).
The arrows indicate when the injections were made.

In a preliminary experiment two rabbits were injected with 0·1 g. Bayer 205 per kg., a dose which is, of course, considerably greater than that normally given to man. Blood samples were removed before the injection and after 5 min., 30 min., 1, 3, 6 and 24 hr., and later at fairly regular intervals. One of the rabbits died (as a result of an unrelated infection) 11 days after the injection, but the other was studied for a total period of 7 months. The results in Table III, show that 6 or 7 months after the injection there is still a determinable and significant amount of the drug in the blood plasma.

In a second experiment two groups each of 3 rabbits were taken. Each rabbit in the first group (group A) received a single dose of 0·14 g. Bayer 205 per kg. The rabbits of group B received over a period of 32 days five injections, each of 0·028 g. Bayer 205 per kg. (equivalent to 2 g. for a 70 kg. man). Determinations of the plasma Bayer 205 were made for 6 months after the first injections. The results of these experiments (Table IV and Fig. 1) indicate that, with a series of injections of small amounts of the drug, the amount present in the plasma is greater, over the major portion of the experiment, than it is when the same total amount of Bayer 205 is injected in one dose.

In another experiment, Antrypol (B.D.H.) was injected into a dog, with a view to finding out whether this preparation behaved exactly like Bayer 205, and partly with the object of measuring the rate of its excretion in the urine. The dog received an intravenous injection of 0·03 g. per kg. and a similar dose 37 days later. Blood samples were taken before the first injection and for a total period of 14 weeks, and the whole of the urine voided during the experiment was collected. For reasons given earlier in this paper, no reliance can be placed on the actual figures for urinary Bayer 205, but with this reservation, it can be stated that they indicate that fairly appreciable amounts of the drug are eliminated in the urine during the first few days. After this initial period, the amount present in the urine is so small that it is less than the variation in the blank values (the "Bayer 205" values for normal urine). The results obtained with the blood samples (cf. Table V) show that Antrypol behaves like Bayer 205, and that two injections of a small amount are sufficient to maintain a significant amount of the drug in the plasma for a period of three months.

Table V. *Amount of Antrypol (determined as Bayer 205) in the plasma of a dog following two intravenous injections of the drug*

Weight of dog: 4·5 kg.
Injections: 0·13 g. Antrypol at the start and 0·13 g. after 38 days.

Time after injection	17 days	37 days	53 days	14 weeks
Antrypol in plasma (mg. per 100 ml.)	2·7	0·8	1·2	1·0

Discussion

For prophylactic purposes it is obviously desirable that the blood Bayer 205 level should remain above a certain, but at present unknown, minimum value. With the method of determination of this drug described in this paper it is hoped that it will be possible in the future to obtain some figure, even if it is only approximate, for the minimum *in vivo* trypanocidal concentration for various species of trypanosomes.

After the method of determination of the drug had been established, the first experiment of the present investigation was designed to give a general idea of the length of time that injected Bayer 205 remains in the body. In this but no other experiment, the dose of Bayer 205 was distinctly higher than the

equivalent of a normal single dose for man. The plasma Bayer 205 level of the injected animals fell very rapidly during the first 6 hr. and thereafter the fall was less rapid. 19 weeks after the injection the plasma contained Bayer 205 to the extent of 2 mg./100 ml. and after 23 weeks 1·2 mg./100 ml. (Table III). As will be mentioned below, it is possible that even in these low concentrations the drug may have trypanocidal effects.

Other experiments were concerned with a comparison of the effects produced by a single large injection with those of a series of smaller doses. The results given in Table IV and Fig. 1 are in favour of the series of injections, and the differences are certainly significant in the important part of the experiment where the plasma Bayer 205 level has fallen to fairly low values; e.g. in the period 7–17 weeks after the first injection. Experiments are now being carried out on man and on rabbits to attempt to establish this superiority of a series of injections. Partly on theoretical grounds and partly on the results of these investigations, it might tentatively be suggested that the best results might be obtained by injecting a fairly high initial dose with smaller doses (e.g. 1 g. Bayer 205 to a 70 kg. man) one week later and subsequently at longer intervals. After each injection there is rapid excretion of a considerable amount of the injected drug, but every subsequent injection, if the delay is not too long, appears to build up a higher "persistent" plasma Bayer 205 level. In this connection it is of interest to note that Duke [1934], as a result of his work with human volunteers and with monkeys exposed to the bites of infected tsetse flies, suggests that the administration of a second dose 3 weeks or so after the first injection enhances the protective effect of Bayer 205.

These investigations have not yet been concerned with the nature of the trypanocidal action of Bayer 205. There still appears to be some doubt as to how this drug causes the death of trypanosomes when it is injected into an infected animal. The *in vitro* trypanocidal action does not seem sufficiently high to account for its powerful *in vivo* action, and it has been suggested that it has an opsonin-like effect on the trypanosomes [Reiner & Köveskuty, 1927; Jancsó & Jancsó, 1934, 2]. The concentration of Bayer 205 needed to kill trypanosomes *in vitro* within 24 hr. at 37° is about 62 mg./100 ml. [Yorke *et al.* 1931]. Jancsó & Jancsó [1934, 1] report, however, that a concentration of 1·7 mg./100 ml. is sufficiently strong to kill trypanosomes and that a trypanocidal action is exerted in concentrations as low as 1·25 mg./100 ml. If the latter figures are accepted, the results recorded in the present paper would suggest that a series of five injections of a "normal" dose of Bayer 205 may give protection for a period of at least 5 months or about 4 months after the last injection. It should be pointed out, however, that Hawking [1937], working with *T. rhodesiense*, has recently reported that an *in vitro* concentration of 125 mg./100 ml. of Bayer 205 is required to kill 100% of the trypanosomes although the majority of the organisms may be killed by 31 mg./100 ml.

Strong experimental support for the "opsonic theory" is offered by these investigations of Hawking [1937], for it has been shown that trypanosomes which have been exposed for more than 2 hr. to a concentration of 10 mg./100 ml., a concentration considerably below that required to kill the trypanosomes, are incapable of infecting mice. Exposure of the trypanosomes to 1 mg./100 ml. of Bayer 205 is occasionally but not usually sufficient to cause loss of infective power. From these results, and when the relatively weak *in vitro* trypanocidal power of Bayer 205 is considered, it seems probable that this drug destroys trypanosomes in the animal body in part at least by an indirect action, possibly by a sensitizing mechanism. Whether the special prophylactic

properties of Bayer 205 are due to its direct action on the trypanosomes or to some indirect action, it must be remembered that the period of exposure of the organisms to the drug is very much longer in the body than it is in *in vitro* experiments. Where a low concentration of the drug is found *in vitro* to possess trypanocidal or harming effects, it is not improbable that even lower concentrations will be effective in the body.

One of the most interesting results of the present investigations is the length of time that Bayer 205 remains in the body. This retention of the injected drug has been noted previously by several authors [for the literature see Findlay, 1930], but in most cases no real estimate of the amount present in the blood could be given and the duration of the experiment was usually comparatively short. An appreciable amount of Bayer 205 is excreted in the urine during the first week or so after the injection but the remainder is excreted very slowly. Several possible explanations might be offered to account for this retention; for example, there might be storage in the liver or some other organ (although Lang [1931] states that there is no removal by the liver), or there might be combination with proteins or some other constituents of the blood. The inhibitory effect on blood coagulation and the anti-complementary power of Bayer 205 suggest that it is capable of combining with certain blood proteins or protein complexes, but further speculation about the cause of the retention of the drug in the body will be left to a subsequent publication.

Summary

1. A method, based on that introduced by Lang [1931], is described for the determination of Bayer 205. The Bayer 205 is hydrolysed by strong HCl and the amines produced are diazotized and coupled with methyl-α-naphthylamine. The red colour produced is compared colorimetrically with those obtained from standard solutions of hydrolysed Bayer 205.

2. Amounts of Bayer 205 as low as 0·2 mg./100 ml. in water (or a quantity of 0·004 mg.) can readily be detected, and accurate determinations made when the concentration exceeds 0·6 mg./100 ml. With blood plasma, amounts greater than 0·6 mg./100 ml. can be determined with sufficient accuracy. Owing to a high and variable blank value, it has not yet been possible to adopt this method for the determination of Bayer 205 in urine.

3. Antrypol (B.D.H.) behaves exactly like Bayer 205 when hydrolysed and determined by this method. The rates of hydrolysis of these two preparations by HCl are practically identical.

4. After intravenous injection of Bayer 205 into rabbits and dogs, the amount of the drug in the plasma falls rapidly during the first few days, owing in part to excretion in the urine, but after this initial period the diminution is less rapid.

5. Five or six months after the intravenous injection into a rabbit of a relatively large dose of Bayer 205 (0·1 g. per kg.) there is still a significant amount of the drug in the plasma (at least 1 mg./100 ml.). Two injections, each of 0·03 g. per kg., into a dog resulted in a plasma Bayer 205 level of 0·8 mg. or more per 100 ml. for 14 weeks after the first injection.

6. Preliminary experiments have shown that rabbits which have received several smaller doses of Bayer 205 over a period of a few weeks, have more Bayer 205 in their plasma in the later stages of the experiment than have rabbits which received one injection of the same total amount. In these experiments, five injections each of 0·028 g. per kg., maintained the average level of Bayer 205 above 4 mg./100 ml. of plasma for about 9 weeks after the first injection, and after 20 weeks the level was still above 1 mg./100 ml.

7. The significance of these small amounts of Bayer 205 in the plasma is discussed in relation to the figures given by other authors for the minimum *in vitro* trypanocidal concentration of this drug. In view of the recent finding of Hawking [1937], that concentrations of 10 mg./100 ml. and sometimes as low as 1 mg./100 ml. destroy the power of trypanosomes to infect mice, it is suggested that the amount of Bayer 205 found in the plasma of an animal several months after a course of injections may be sufficient to account for the marked prophylactic action of this drug.

The authors take this opportunity of expressing their gratitude to the Medical Research Council for personal grants to two of them (W. E. G. and later W. G. D.) and for a grant which has, in part, covered the expenses of these investigations. Thanks are also tendered to Messrs B.D.H. for a supply of Antrypol, and to Dr E. H. Rodd of the Dyestuffs Group of I.C.I., Ltd., and Prof. F. M. Rowe, of the University of Leeds, for generous samples of various coupling agents.

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XII. NOTE ON THE QUANTITY OF THEOBROMINE IN THE MILK OF COWS FED ON A DIET INCLUDING THIS ALKALOID

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(*Received 24 November 1937*)

UNTIL the discovery by Knapp & Coward [1934] that cacao shell is rich in vitamin D, this by-product of the cocoa and chocolate industry never achieved more than a casual interest as an accessory cattle food. Kon & Henry [1935] reported that the feeding of 2 lb. of shell daily for a month during the winter raised the vitamin D content of the milk of stall-fed cattle to the normal summer level, whilst Golding & Burr [1937] found that the fat content of these milks was increased during the period of shell feeding. Because of these facts it appears probable that cacao shell will occupy a much more prominent place as a cattle fodder than it has done in the past.

Cacao shell contains approximately 1% of theobromine, an alkaloid valued by pharmacologists for its properties as a stimulant and diuretic. It is natural to enquire how much, if any, of this alkaloid reaches the milk of cattle fed with shell. An opportunity to throw some light on this matter was recently presented when an experiment was conducted at this Institute to determine whether the increase in the milk-fat yield, which had been observed when cacao shell was fed, was due to the theobromine alone. In this experiment, theobromine was fed to a small group of dairy cows for a period of 3 weeks; the daily weight administered was 9 g., which is the quantity contained in 2 lb. of cacao shell. Samples of milk were taken from three cows from this group on the last day of the feeding and an estimate made of their theobromine contents. The results obtained (Table I) and the chemical procedure adopted are given below.

Table I

Animal	Average daily milk yield lb.	Yield on last day of experiment lb.	"Theobromine" in milk mg. per l.
Profit	35·06	$8\frac{3}{4}$	4·71
Lead 46	19·44	$6\frac{1}{2}$	2·05
Sainfoin	17·75	6	4·72

Method of analysis. The method adopted was that of Kunz [1935]. The proteins in 1 l. of milk were precipitated with trichloroacetic acid. After filtration, the filtrate was neutralized with ammonia, conc. *in vacuo* to about 100 ml., seeded with lactose crystals, and left for 24 hr. The crystals of lactose which formed were removed by centrifuging, the solution made slightly alkaline with ammonia and the precipitate again removed by centrifuging. The solution was conc. to 40 ml. and continuously extracted with chloroform for at least 8 hr. About 10 ml. of water were floated on the surface of the $CHCl_3$ which was then distilled off. To the resulting aqueous solution was added excess 30% phosphotungstic acid, the mixture was left overnight in an ice chest, and the bases regenerated from the precipitate by means of baryta. After removal of excess

Ba with CO_2, the solution was boiled, filtered and conc. to 20 ml. 1 ml. each of 10% acetic acid and satd. sodium acetate were added, the solution was heated to boiling and 1 ml. each of 40% $NaHSO_3$ and 13% $CuSO_4$ were added. After boiling 5 min., it was filtered directly into the extraction apparatus and extracted for 5 hr. with $CHCl_3$. The extract was transferred to aqueous solution as before and brought to 25 ml. From the N content of this solution, determined by micro-methods, the concentration of theobromine was calculated (1 ml. $N/100$ acid ≡ 0·4499 mg. theobromine). This figure would include any caffeine that might be present, as it was considered unnecessary further to complicate the analysis with another precipitation.

Controls. A blank determination on normal milk gave a "theobromine" content as 0·99 mg. per l. Kunz [1935] obtained a blank of 0·2–0·4 mg. per l. as caffeine in woman's milk, but gives no similar figure for theobromine. In normal cow's milk to which had been added 10·37 mg. theobromine per l. I could detect only 6·89 mg. per l. using the technique described above.

Isolation and identification of theobromine in milk. Seven hundred ml. of milk from Sainfoin were treated according to the above method as far as the second chloroform extraction, when the residue after evaporation of $CHCl_3$ amounted to 3·9 mg., equivalent to a concentration of 5·57 mg. per l. of milk, which exceeds the value calculated from the N-determination by only 0·85 mg. per l.

An attempt was made to recrystallize this residue from water, but the crystals so obtained were of irregular shape although of the same general (minute) size as theobromine crystals. They gave a definitely positive reaction to the following test for theobromine, caffeine and theophylline: To 0·5 mg. theobromine on a watch-glass add 2 drops of a mixture of conc. HCl diluted with 2 vols. water. Boil, add 5 drops Br-water, and evaporate carefully to dryness. Dissolve the residue in 5 drops water, add 1 drop 1% $FeSO_4$, place 1 drop *N* ammonia by the side of the solution, and allow slowly to mix. An intense dark blue coloration forms, which quickly fades on standing or with excess ammonia.

Discussion

It is evident that a very small quantity of theobromine has passed into the milk of these cows, the maximum concentration being approximately 7 mg. per l.

The British Pharmaceutical Codex and Squire's Companion to the British Pharmacopoeia give the medicinal dose of theobromine as 0·3–0·6 g.; taking the case of a child of under 12 months, for which the dose would be about 1/12th of that prescribed for an adult, the minimum dose is contained in $6\frac{1}{4}$ pints of this milk. Perhaps a better idea of the harmlessness of this concentration of theobromine is obtained when it is considered that the average plain eating chocolate contains 0·3% and that it is not uncommon for $\frac{1}{4}$ lb. of this chocolate (containing 0·3 g. of theobromine) to be eaten by an adult at one time. It is unlikely, therefore, that the proportion of theobromine appearing in these milks could have any noticeable effect on the most delicate of constitutions.

One would expect both the health of the animal and the period of feeding to influence the proportion of alkaloid appearing in the milk, and a more comprehensive experiment on these lines seems desirable.

SUMMARY

9 g. of theobromine were fed daily to each of three cows for a period of 3 weeks. The maximum concentration of theobromine appearing in the milks yielded on the last day was approximately 7 mg. per l., an amount which may be presumed to be entirely innocuous.

I am very glad to acknowledge the kindly advice and assistance given to me during the progress of this work by Captain J. Golding.

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XIII. POTASSIUM CONTENT AND CELL VOLUME OF CENTRIFUGED ERYTHROCYTES

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(*Received 19 November 1937*)

Danielli [1937] remarks that centrifuging bloods of certain species causes a loss of up to 40% of cell potassium from the erythrocytes. He has not worked with human erythrocytes and as all analyses of human cells depend directly or indirectly on their separation from plasma by centrifuging, the present writers have endeavoured to find out if such treatment causes any change in the potassium content or volume of these cells.

Potassium content. Rapidly sedimenting bloods from cases of rheumatoid arthritis were heparinized and divided into three portions. One was allowed to settle and the other two were centrifuged for 10 min. at 4500 and 10,000 rev. per min. respectively. After centrifuging, any potassium expressed from the cells would appear in the supernatant plasma and lest any local accumulation might occur in the layer immediately above the packed cell columns, the whole of the plasma was carefully mixed with a glass rod and then recentrifuged. In the case of the sample allowed to settle naturally, the clear plasma was removed with a pipette and any traces of suspended cells were removed by centrifuging. The potassium contents of the three separated specimens were estimated by the method of Kramer & Tisdall [1921] (Table I).

Table I

	Potassium contents (m.eq. per 1000 ml.)			
Rev. per min.	Exp. 1	Exp. 2	Exp. 3	Exp. 4
0	4·2	4·45	3·91	3·01
	—	4·51	3·94	2·91
4,500	3·6	4·73	3·88	3·25
	—	4·73	3·94	3·27
10,000	4·0	5·22	3·70	3·73
	—	5·12	3·91	3·75

Increase in plasma K as a result of centrifuging varies between 0 and 25%. Since the erythrocyte contains 25 times as much potassium as does plasma, it follows that less than 1% of cell K is lost as a result of centrifuging—provided that one assumes that no loss of cell water occurs simultaneously. And in fact loss of cell water could not account for the constancy of plasma K, since in order to mask a loss of 1% of cell K a simultaneous outflow of 35% of cell water would have to occur and to compensate for the loss of 2%, the cell would have to be desiccated assuming that water occupies 70% of the cell volume. It may therefore be accepted that no loss of cell potassium occurs as a result of centrifuging. On the other hand an apparent change of cell potassium might occur owing to expression of cell water; it seems unlikely that centrifuging could increase the amount of water in cells. In the following section it will be shown that cell volume remains practically unaltered during centrifuging.

Method 1. A solution of pure haemoglobin was prepared [Adair & Adair, 1934] and rendered isotonic with sodium chloride. A small amount of the coloured solution was added to a rapidly sedimenting blood. The mixture was set up in four graduated tubes and allowed to settle for 6 hr., after which time two of the tubes only were centrifuged. The supernatant haemoglobin-plasma was removed and a measured portion set aside as a colour standard. All the residual coloured plasma were then carefully washed out of the containing tubes without disturbing the cells. The latter were suspended in saline solution and in this way haemoglobin-plasma was washed out from between the cells and on recentrifuging appeared in the supernatant saline. The volume of this saline was measured and its haemoglobin content compared in a colorimeter with the standard solution. In this way the volume of intercellular haemoglobin-plasma is obtained. If then one subtracts the respective volumes of intercellular fluids from the corresponding volumes of settled and centrifuged cells one obtains the absolute volume of cells in each case.

Method 2. Here, plain heparinized blood was used, one portion being allowed to settle and the other being centrifuged. In both cases supernatant plasma was removed and intercellular plasma washed out of the erythrocyte columns by suspension in saline and recentrifuging. The protein content of this supernatant plasma was then compared with that of the original supernatant plasma, the colour reaction of Wu & Ling [1927] being used. Figures in Table II are expressed in volumes % of original suspension.

Table II

	Settled cells vol. %	Inter-cellular fluid vol. %	Absolute cell vol. %	Centrifuged cells vol. %	Inter-cellular fluid vol. %	Absolute cell vol. %
Method 1: tube 7 mm. bore						
Exp. 1	42	10	32	35	2	33
Exp. 2	41·5	9	32·5	35	1·5	33·5
Exp. 3	43	15	28	28·5	1·5	27
Exp. 4	44·5	10	34·5	33·5	2	31·5
Exp. 5	46·5	12·5	34	38	2·5	35·5
Method 2: tube 3 mm. bore						
Exp. 1	47	11·5	35·5	37	3	34
Exp. 2	55	11·5	43·5	45	1·5	43·5
Exp. 3	42·5	10	32·5	32·5	2	30·5
Exp. 4	42·5	7·5	35	34	1·5	32·5

It will be seen that with one exception the absolute volume of cells after settling differs little from the absolute volume after centrifuging, the variations lying between $-4{\cdot}5$ and $+7$ %—differences which are within the range of experimental error.

SUMMARY

Centrifuging human erythrocytes, at least in rapidly sedimenting blood, causes little or no change in the base or water content.

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XIV. INVESTIGATIONS ON THE NATURE OF CALCIUM BOROGLUCONATE

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(*Received 16 November 1937*)

For some time before 1935, solutions of calcium gluconate were employed in the treatment of acute hypocalcaemia, notably milk fever in cows. Injection of this substance was, however, often attended by serious local reaction, due chiefly to the low solubility of the gluconate. Dryerre & Greig [1935] having confirmed the observation of Hayden [1934] that boric acid greatly increases the solubility of calcium gluconate, advocated the use of calcium "borogluconate" in preference to calcium gluconate in cases of milk fever. The "borogluconate" is now widely employed with satisfactory results.

The name calcium "borogluconate" was suggested by Dryerre & Greig for the substance formed on evaporation of an aqueous solution containing five parts by weight of calcium gluconate and one part of boric acid, these being the proportions used for injection. The authors observed that the substance differed from boric acid and from calcium gluconate in that it gave no green flame coloration and possessed a different crystal structure. It was also about thirty times as soluble as calcium gluconate at 15°.

Apart from these facts, nothing was known about the nature of this "borogluconate" and it was with a view to obtaining more definite knowledge of the substance that the present experiments were undertaken.

Some of the substance was prepared according to Dryerre & Greig, evaporation being carried out under reduced pressure at 40° and also over a boiling water bath: there appeared to be no difference between the two specimens in the course of the experiments. Titration experiments on the "borogluconate" so obtained showed that in aqueous solution all the boron was present as free boric acid: the compound, if it existed as such, therefore seems to be wholly split up into its components in aqueous solution. This was borne out by the fact that when an aqueous solution of the "borogluconate" was partly re-precipitated by the addition of ethyl alcohol, the precipitate and the solution contained entirely different proportions of boric acid.

The fact that the substance exists as a mixture of its constituents in aqueous solution, that it is almost insoluble in alcohol and other solvents and that proper melting points for gluconate and "borogluconate" cannot be obtained, raised the difficulty of devising a method to show whether, in the solid state, the "borogluconate" is an actual compound or merely a mixture. This obstacle was overcome by employing a distillation method, ethyl (or methyl) borate being volatile and easily distilled. If a mixture of calcium gluconate and boric acid is distilled with ethyl alcohol, all the boric acid is driven off as the ester. This was carried out with a mixture of the two substances in the proportions employed by Dryerre & Greig, and after distillation for a short time it was found that the residue contained no boric acid. On the other hand, if some "borogluconate" is prepared by evaporation of a solution containing the same proportions, and subjected to distillation with alcohol, the residue still retains all

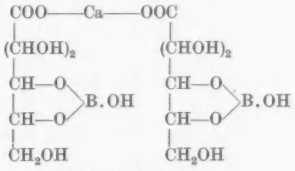
of the boric acid. Obviously, then, the "borogluconate" is not merely a mixture, the boric acid being attached in some way to the gluconate molecule. A flame test experiment on the same lines bears this out. If a mixture of gluconate and boric acid is ignited with ethyl (or methyl) alcohol, a brilliant green coloration is imparted to the flame, whereas with the "borogluconate" no such coloration is obtained, unless some conc. H_2SO_4 is also present.

Considering the structure of the "borogluconate", the most likely possibility would seem to be the attachment, by loss of water, of the boric acid to a pair of *cis*-hydroxyl groups in the gluconate molecule. This is in accordance with the formulation first proposed by van't Hoff [1870] and more recently supported in numerous papers by Böeseken and his co-workers at Delft.

The proportions of calcium gluconate and boric acid employed by Dryerre & Greig do not coincide with those required for a compound of the gluconate with one or more molecules of boric acid. Accordingly experiments were carried out to determine, if possible, the amount of boric acid required to saturate the gluconate molecule.

A solution containing calcium gluconate and a large excess of boric acid was evaporated to dryness: the substance was then distilled for a considerable time with ethyl alcohol. The boric acid in the residue was then estimated and was found to correspond almost exactly with the amount required, on the van't Hoff formulation, for a compound of calcium gluconate with two molecules of boric acid. Repeated distillation of the residue failed to alter the proportion of boric acid. The compound obtained after distillation showed no green flame coloration with alcohol in the absence of conc. H_2SO_4.

It may therefore be assumed that calcium borogluconate proper is a compound of one molecule calcium gluconate and two molecules boric acid and the following may be suggested as a probable formula:



i.e. calcium diborogluconate.

Experimental

Estimation of boric acid. This was carried out in aqueous solution by titration with $N/20$ NaOH in the presence of excess glycerol, using phenolphthalein as indicator. The glycerol was added gradually during the titration until the pink colour no longer disappeared. Calcium gluconate, which was present in all the solutions under examination, was quite neutral to phenolphthalein as was the pure glycerol used. The gluconate was a "Sandoz" product.

Preparation of calcium "borogluconate". 6 g. calcium gluconate and 1·2 g. boric acid (H_3BO_3) were dissolved together in distilled water, warming to facilitate solution: the solution was then filtered and evaporated to dryness in a boiling water bath (or under reduced pressure).

Removal of free boric acid by distillation with alcohol. 1–2 g. of material were usually employed. The substance was put into a small (100 ml.) distilling flask with about 40 ml. absolute alcohol, and distilled on a water bath: more alcohol was added when the level became low and the distillation repeated. Two such

distillations were usually sufficient to remove all the boric acid. To estimate the remaining boric acid, the alcohol was filtered off from the residue, which was then dried in a vacuum desiccator, and a weighed quantity dissolved in water and titrated against standard alkali as described above.

Preparation of calcium borogluconate proper. This can be prepared by accurately weighing out the required proportions of calcium gluconate and boric acid (1 mol. : 2 mol.) but a more satisfactory method is to have present a slight excess of boric acid, and after evaporation of the solution to dryness, expel the excess by distillation with alcohol.

The calcium borogluconate is very soluble in water and in 20% solution has *p*H approximately 3·5, as compared with 3·7 for the "borogluconate" of Dryerre & Greig. An aqueous solution of a mixture of gluconate and boric acid (1 : 2 mol.) also has *p*H 3·5.

Summary of experimental results

Substance under examination	% H_3BO_3 Found	% H_3BO_3 Calculated
Prepared "borogluconate"	16·53	16·67
Mixture of gluconate and boric acid (5 : 1)	16·50	16·67
Mixture after distillation with alcohol	0·00	—
"Borogluconate" after distillation with alcohol	16·80	—
Borogluconate proper, prepared with excess H_3BO_3 and distilled with alcohol	25·90	13·60 (1 mol.) 25·73 (2 mol.) 36·00 (3 mol.)

It is to be noted that calcium gluconate and boric acid mutually increase each other's solubility in water. A 6% boric acid solution, for example, is quite stable at 15° in the presence of calcium gluconate, whilst a 25% gluconate solution is stable in the presence of 2% H_3BO_3.

Summary

Calcium borogluconate proper is a definite compound formed from one molecule of calcium gluconate and two molecules of boric acid by loss of water. In aqueous solution it behaves as a mixture of these two substances.

A formula is suggested; the substance may be called calcium diborogluconate. The "borogluconate" of Dryerre & Greig is a mixture of calcium mono- and di-borogluconates.

The authors acknowledge their indebtedness to the Director of the Institute, Dr J. Russell Greig, for his great interest in this work and for much valuable criticism.

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XV. MUCONIC ACID AS A METABOLIC PRODUCT OF BENZENE

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Jaffé [1909] isolated muconic acid from the urine of rabbits and dogs to which benzene had been administered. The amount was approximately 0·3% of the benzene given, but Jaffé considered that a larger amount was formed in the tissues, possibly as much as 25–30%, by the opening of the benzene ring, because he found that injected muconic acid was metabolized, only about 1% being recovered in the urine. Fuchs & Soos [1916] found muconic acid in the urine of patients who had been treated for leucaemia with benzene. The yield was also small (0·11%).

Higher yields, corresponding to about 3·7% of the benzene administered, have been recorded by Thierfelder & Klenk [1924], but not every investigation has confirmed Jaffé's work. Neumaerker [1923] failed, in spite of using an improved method of extraction, to separate any muconic acid from the urine of a rabbit which had been injected subcutaneously with 3 g. of benzene. The question which needed further study was that of the origin of the muconic acid, because, apart from some uncertainty whether it is always produced, there was the important consideration, based on the work of Böeseken and his colleagues [1929; 1932], that one would expect the *cis-cis* form of the acid to be formed, whereas the evidence indicated that the acid isolated from urine was the *trans-trans* form. Böeseken & Slooff [1929] have remarked, "If it should really be true that on addition of benzene to the food, *trans-trans* acid was separated by the test animals, this is more likely the consequence of a reduction and dehydration, e.g. of galactose, as the grouping of the atoms of this sugar and the closely allied mucic acid renders the formation of *trans-trans* muconic acid possible." The suspicion that the muconic acid in the urine might arise not directly from the benzene but as a result of disturbances of metabolism caused by it seemed to be strengthened by the discrepancies regarding the fate of the acid after it is injected into animals. Jaffé [1909] recovered less than 1%; Neumaerker [1923] as much as 53%; Mori [1918] the very high yield of 73%. The last-mentioned investigator was inclined to doubt that the acid was formed after giving benzene.

Experimental

Preliminary experiments indicated that the methods already described for isolating muconic acid from urine were unsatisfactory. Recovery of added acid was always poor.

The basis of a better method was found in the fact that the acid, although very slightly soluble in dry alcohol or acetone is very readily soluble in both solvents in the presence of moisture and acid. It is also very soluble in a wet acetone-ether mixture.

trans-trans Muconic acid was prepared by the bromination of adipic acid chloride [Stephen & Weizmann, 1913]. The dibromoacid chloride was converted

into the ethyl ester [Ingold, 1921] and by debromination and hydrolysis into the muconic acid, decomp. p. 301°.

The *cis-cis* acid was prepared by the oxidation of phenol by peracetic acid [Böeseken & Engelberts, 1932]. It decomposed at 186°.

Recovery trials

(*a*) trans-trans *Muconic acid.* 0·2 g. of the *trans-trans* acid as the Na salt was added to 50 ml. of rabbit urine, acidified to Congo red with HCl and the volume made up to 75 ml. Equal volumes of acetone and ether were added and the mixture well shaken. On standing the layers separated, the upper nearly colourless, the lower red. The former was separated and the solvents removed. The residual aqueous fluid contained a small amount of brown suspended solid which went into solution on adding an excess of ammonia. Boiling with norite decolored the solution and on acidification a white precipitate was formed immediately. The crude product melted with decomposition at 299°. The yield was 84%.

The method was found suitable for the recovery of smaller amounts, and could detect as little as 10 mg./100 ml.

Exp.	Wt. of muconic acid added to 50 ml. of normal urine g.	Wt. of muconic acid recovered g.	Recovery %
a	0·4	0·3549	88·7
b	0·2	0·1741	87
c	0·1	0·0840	84
d	0·05	0·0378	75·6
e	0·025	0·0186	74·4
f	0·010	0·0057	57
g	0·005	Recovered a trace of impure substance decomp. 283°	

The crude preparations of the acid were often purified by sublimation at 0·001 mm. Quite small quantities could be obtained in a pure condition by this procedure.

(*b*) cis-cis *Muconic acid.* The first experiments on the recovery of added *cis-cis* acid indicated that our method of extraction tended to convert it into the *trans-trans* form. Later it was found that provided that the precaution was taken of evaporating the alcohol-ether fractions at low temperature the *cis-cis* acid could be recovered from normal urine.

0·3 g. *cis-cis* muconic acid, as the Na salt, was added to 400 ml. of "normal" rabbit urine and the solution kept for 24 hr. The urine was made acid to Congo red with HCl and an equal volume of acetone (400 ml.) added and the mixture shaken vigorously. Then 400 ml. of ether were added and the mixture again shaken vigorously. After a short time two layers separated. The lower layer was run off and again extracted with a mixture of 100 ml. of acetone and 100 ml. of ether. The lower layer obtained again was extracted with another mixture of 100 ml. of acetone and 100 ml. of ether. It was run off and again extracted as before. All four acetone-ether extracts were combined and made alkaline with ammonia, whereupon an aqueous phase separated at the bottom. The volume of this lower layer was 120 ml. It was acidified with HCl and kept overnight. No precipitate formed. It was therefore extracted four times with acetone-ether mixtures as before, using smaller volumes of solvent. The four extracts were combined and made alkaline with ammonia, whereupon an

aqueous phase separated. This was run off, and the acetone and ether dissolved in it were removed by evaporation under reduced pressure in the cold. It was then acidified with HCl and kept overnight. A precipitate formed which was collected, washed with water and dried *in vacuo* over P_2O_5. The yellow powder was dissolved in dilute ammonia and reprecipitated.

The purified material melted at 183° and was identified as the *cis-cis* acid. The recovery was 56%. Satisfied, therefore, that both acids could be separated from rabbit urine we proceeded to make experiments on the effect of the administration of benzene. The adult rabbits were given the hydrocarbon dissolved in olive oil by means of a stomach tube. When 0·25 or 0·5 ml. of benzene was given over a period of 8–10 days the urine did not yield any muconic acid. After giving 2 ml. dissolved in 5 ml. of olive oil for 6 days a few mg. of impure *trans-trans* acid were isolated. When the dose was raised to 3 ml. of benzene daily the results were satisfactory. From the urine representing 5 days' dosing at this level 146 mg. of acid were isolated. It was the *trans-trans* form and no trace of the *cis-cis* isomeride was found. The yield in several experiments was about 0·4%; a figure agreeing reasonably well with Jaffé's original observations.

It was now necessary to satisfy ourselves of the fate of the two isomerides when injected into the body.

Injection experiments

(*a*) trans-trans *Acid*. Rabbits were injected subcutaneously with 0·89 of the acid given in 20 ml. of an aqueous solution of the Na salt.

The urines were collected during 72 hr. and examined by the method just described. By examination of each day's urine it was found that practically the whole of the muconic acid excreted was present in the samples taken over the first 24 hr. Only a few mg. were isolated from the urine of the second day and none from that of the third.

Rabbit	Muconic acid injected g.	Volume of urine ml.	Reaction of urine to litmus	Muconic acid in urine g.	Recovery %
A	0·8000	104	Neutral	0·4807	60
B	0·8000	122	Alkaline	0·5101	63·8
C	0·8000	87	,,	0·4240	53
A	0·8000	115	,,	0·4520	56·5
B	0·8000	153	,,	0·4015	50·2
C	0·8000	107	,,	0·4183	52·3
				Average	56

The acid was in all cases unchanged *trans-trans*. These yields are much better than Jaffé obtained and more in agreement with those recorded by Mori.

(*b*) cis-cis *Acid*. Two rabbits were given 0·8 g. of the *cis-cis* acid as the Na salt. It was injected subcutaneously in 20 ml. of water. The acid recovered from the urine was also *cis-cis* and the yields were respectively 47·5 and 45·3%. No *trans-trans* acid was isolated and it is important to remember that had it been present its smaller solubility would probably have led to its detection before the *cis-cis* acid separated out.

Origin of trans-trans *acid in "benzene" urine*

We failed to isolate any *cis-cis* acid from the urine of the animals which had received benzene. As the experiments on the recovery of injected *cis-cis* acid gave nearly a 50% yield and provided no evidence of conversion into the *trans-trans* we were inclined to think that had benzene given rise to the former acid it would have been found in the urine. One thing had, however, aroused our suspicion. The urine voided by rabbits given rather large doses of benzene is rich in phenolic substances and darkens.

In one experiment on the recovery of added *cis-cis* acid from urine it was found that conversion into the *trans-trans* form occurred rather readily when "benzene" urine was used. No evidence of conversion was obtained when the process was applied to normal rabbit urine.

0·4 g. of *cis-cis* muconic acid, as the Na salt, was added to

(1) 400 ml. of "normal" rabbit urine.
(2) 400 ml. of "benzene" rabbit urine.

Both were kept for 24 hr. Each was made acid to Congo red with HCl and extracted four times with acetone-ether mixtures, as previously described. The acetone-ether extracts were combined and made alkaline with ammonia, whereupon an aqueous phase separated. The lower aqueous phase was run off and evaporated on the water bath to small bulk, the liquid being kept alkaline with ammonia during the course of the evaporation. The solution was cooled, strongly acidified with HCl and kept overnight. A precipitate formed. This was collected, washed, and dried *in vacuo* over P_2O_5.

(1) Decomposition point was 181° (*cis-cis*).
(2) Decomposition point was 293° (*trans-trans*).

By modifying the process so as to eliminate the heating of the extracts, it was found possible to recover added *cis-cis* acid in the presence of *trans-trans* acid in "benzene" urine.

(*a*) *Urine without addition of* cis-cis *muconic acid.* An impure substance was isolated (about 19 mg.) the decomposition point of which was 291°. This, therefore, was *trans-trans* muconic acid.

(*b*) *Urine to which* cis-cis *muconic acid had been added.* When the second aqueous solution was obtained, the acetone and ether dissolved in it were removed under reduced pressure in the cold. It was then acidified with HCl, and after about an hour a small precipitate formed. This was collected and dried *in vacuo* over P_2O_5. The weight of this precipitate was 28 mg., and its decomposition point 293°. This was *trans-trans* muconic acid.

The filtrate after scraping the sides of the vessel with a glass rod began to form a second precipitate the amount of which gradually increased. After a few hours this precipitate was collected, washed with water and dried *in vacuo* over P_2O_5. The weight of the crude substance obtained was 0·33 g. and its decomposition point was 176°. It was further purified by reprecipitation from its ammonium salt. The decomposition point of the purified product was 184°. It was identified as the *cis-cis* acid. This experiment was repeated four times with the same result.

The evidence seemed to be pointing to a possible action of some substances in "benzene" urine which might under suitable conditions lead to a conversion of the *cis-cis* into the *trans-trans* acid.

To throw light on this question a rabbit was given the usual dose of 3 ml. benzene for two periods of 3 days. It was then injected with 0·22 g. of the

cis-cis acid (Na salt), and the benzene administration continued for 3 days. Finally there was another 3-day control period of benzene. The results were:

Period	Vol. urine ml.	Acid isolated	Wt. mg.
1 benzene	225	*t-t*	58
2 benzene	175	*t-t*	80
3 benzene and *cis-cis* acid	510	*t-t*	107
4 benzene	500	*t-t*	43

The difference between the yields of *trans-trans* acid in the three control periods makes it difficult to decide whether the increased excretion during period 4 is significant. It probably is, because no *cis-cis* acid was isolated whereas from the results on p. 82 a yield of about 100 mg. might have been expected.

Although the evidence is not conclusive it seems to indicate that the *cis-cis* acid may be converted into the *trans-trans* form during the metabolism of benzene.

Why this happens under these conditions but does not in the normal rabbit when the *cis-cis* acid is given cannot yet be explained.

Other substances present in "benzene" urine

The urine of the rabbits to which benzene had been given was examined for other products. Free benzene was detected by using the apparatus devised by Cannan & Sulzer [1924] for the micro-estimation of alcohol in body fluids.

The aspirated air passed through a nitrating mixture composed of equal parts of H_2SO_4 and fuming HNO_3. This fluid was examined after several hours' aeration by applying the colour reaction described by Schrenk *et al.* [1935] and Pearce *et al.* [1936].

The reaction was negative when amounts smaller than 3 ml. of benzene were administered, but with that quantity or with larger doses free benzene was detected in the urine. The amount of phenolic substance rose considerably during the periods when benzene was being given. The results of a typical experiment which show the rise in ethereal sulphates are recorded below.

		mg. per kg. body weight				
		Inorganic S		Ethereal S		
Day	Rabbit	I	II	I	II	
1		93	121	4·9	4·5	5 ml. olive oil daily by stomach tube
2		82·5	121	5·2	1·5	
3		71·5	110·5	3·7	4·1	
4		60·5	84	2·3	6·8	
5		—	—	—	—	
6		28·6	7·5	33	32	3 ml. of benzene in 5 ml. of olive oil daily
7		2·1	43·7	38·6	33·8	
8		31·2	61	34·2	26·5	
9		8·7	32	34·6	29·2	
10		27·8	42·4	35·2	30·7	

The increased excretion of ethereal sulphates was accompanied, as was expected, by a rise in the excretion of glycuronates but this was not actually estimated. Several attempts were made to isolate phenylmercapturic acid from the "benzene" urine but without success. Neither the method used by Bourne & Young [1934] for the isolation of α-naphthylmercapturic acid, nor the preparation of a mercury compound yielded material which could be adequately

purified. Strong evidence that these fractions contained phenylmercapturic acid was obtained by treating them with acids when thiophenol was produced. The production of phenols by the oxidation of benzene made it desirable to ascertain whether muconic acid can be found in the urine after administering either phenol itself or catechol.

The largest amount of phenol which it was found practicable to give a fully-grown rabbit by stomach tube was 0·25 g. in 50 ml. of water. This dose for 4 days produced a dark urine rich in phenolic sulphates and glycuronates but no muconic acid was isolated.

In all probability the dose of phenol was too small for the amount of muconic acid formed, if any, to be recoverable by our methods. The permissible dosages of catechol were even smaller, so it is not surprising that these also failed to yield muconic acid. Negative results were also recorded in an experiment in which 3 g. of tyrosine dissolved in 80 ml. of warm milk were given to a rabbit for 6 days. This confirms Kotake (cited by Mori [1918]).

Discussion

Our results are not wholly conclusive but they tend to support the view that the *trans-trans* muconic acid found in the urine of animals to which benzene has been administered does arise from the hydrocarbon molecule. It is a curious finding for which our work has not provided a satisfactory explanation, that the *cis-cis* acid, which, as Böeseken has pointed out, would be the one directly derived from the opening of the benzene ring, is rather readily converted into the *trans-trans* isomeride in the presence of other products formed when benzene is metabolized. In striking contrast one finds the *cis-cis* acid not converted in the normal organism or in contact with the substances present in normal urine.

We desire to thank Prof. Böeseken for his kindness in providing a specimen of pure *cis-cis* muconic acid and for much helpful information about its preparation.

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XVI. COMBINED ASCORBIC ACID IN PLANT TISSUES

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Several chemical methods have been suggested for the estimation of ascorbic acid. Of these, titration with the dye dichlorophenolindophenol has met with most favour and has been extensively employed. Using this procedure, McHenry & Graham [1935] found that short periods of cooking caused an apparent increase in the titration values of certain vegetable tissues. A similar increase was obtained after acid hydrolysis of the tissues at room temperature. As an explanation of these increases McHenry & Graham postulated the presence in the foods of an ascorbic acid compound, insoluble in the extractants ordinarily used, and from which compound ascorbic acid was freed by hydrolysis.

Confirmation of these results was secured by Ahmad [1935], Guha & Pal [1936], and by Levy [1937]. Van Eekelen [1935] and Mack [1936] were unable to verify these results and both authors believed that the observed increase in titration values was caused by the destruction of "ascorbic acid oxidase". This explanation assumes that an extract of fresh tissue contains reduced ascorbic acid and the oxidase system, the latter of which converts some of the ascorbic acid into dehydroascorbic acid. If the tissue is heated prior to extraction the enzyme is destroyed, no oxidation of ascorbic acid can occur, and the titration value will be higher. Since McHenry & Graham had estimated both reduced ascorbic acid and dehydroascorbic acid in both fresh and heated tissues, this explanation is not applicable to their results. Mack & Tressler [1937] reported the production of reducing substances by heating dehydroascorbic acid in almost neutral solutions and believed these to be physiologically inactive degradation products. They explained the apparent increase of free ascorbic acid after heating to be due to the formation of such products. However, the ascorbic acid liberated from the protein complex is physiologically active, as will be shown later in this paper.

While chemical evidence had indicated that a substance similar to ascorbic acid was released by hydrolysis from certain tissues, the identity of the substance had not been established. In this communication chemical, spectroscopic and biological data are reported, indicating that the substance secured by hydrolysis of the protein complex is ascorbic acid.

Chemical studies

The protein-ascorbic acid compound is soluble in cold water and may be precipitated from a water extract of plant tissue by the addition of trichloroacetic acid or metaphosphoric acid. This is a convenient method for separating combined ascorbic acid from the reduced and reversibly oxidized forms. The precipitate, containing protein and ascorbic acid in combination with protein, is hydrolysed with 0·2–1·0% hydrochloric acid. Subsequent precipitation with the same protein precipitants removes protein, leaving the substance which reduces

phenolindophenol in solution. Such a solution behaves similarly to one of pure *l*-ascorbic acid and gives negative tests for other reducing substances. "Ascorbic acid oxidase" destroys the reducing properties of the solution as does oxidation by chemical reagents. Qualitative and quantitative analyses show that the precipitated material, formerly combined with ascorbic acid, is protein.

Table I summarizes a series of heat treatments of potato tissue, and Table II gives the amounts of ascorbic acid obtained by various extractions of similar

Table I. *Effect of heating treatments on the free ascorbic acid content of potato tissue*

Treatment	Total free ascorbic acid mg. per 100 g.
1. Raw—extraction with CCl_3COOH—$(HPO_3)_4$	14·1
2. Heated 3 min. at 98° under CO_2	19·5
3. Heated 15 min. at 98° without CO_2	11·2
4. Heated 30 min. at 98° without CO_2	6·9
5. Heated 30 min. at 98° with 0·5% citric acid	10·0
6. Heated 30 min. at 98° with $NaHCO_3$ to *p*H 9–11	1·3
7. Heated as above with oxygenation	Nil
8. Dehydrated at 35–40°	5·8

Table II. *Effect of various extraction treatments on the titration values for ascorbic acid*

Treatment	Total ascorbic acid mg.
1. 25 g. potato tissue extracted with cold water	7·4
2. 25 g. potato tissue extracted by heating 3 min. at 98° under CO_2 in distilled water	7·4
3. 25 g. potato tissue extracted with CCl_3COOH after hydrolysis with 1% HCl	7·5
4. 25 g. potato tissue extracted with cold CCl_3COOH—$(HPO_3)_4$	5·4
5. (*a*) 25 g. potato tissue extracted with cold water	7·2
(*b*) 20% CCl_3COOH added to extract and precipitate removed	5·3
(*c*) Centrifuged precipitate hydrolysed with dilute acid	1·8
6. 25 g. potato tissue extracted with cold absolute alcohol	3·5
7. 25 g. potato tissue extracted with absolute alcohol after heating	7·1

tissue. All determinations were made by titration with 2:6-dichlorophenol-indophenol. Samples of tissue were taken from a well mixed, evenly sliced lot to prevent variation in the original ascorbic acid content. As found by Guha & Pal [1936] the heating of an absolute alcohol extract of potatoes causes an increase in titration value. Since "ascorbic acid oxidase" is inactivated by absolute alcohol, and is inhibited by the mixture of trichloroacetic and metaphosphoric acids used in extraction, the increase cannot be due to destruction of the oxidase. Other results in Table II tend to confirm the presence of protein-ascorbic acid.

In Table III the effects of heating various plant tissues on the values found for ascorbic acid after extraction and titration are given. Some tissues show an appreciable increase, others only a decrease after similar treatment. Fruits give a more gradual decrease after heating and in no case an increase. Apparently protein-ascorbic acid occurs only in some vegetable tissues.

Table IV records the amounts of ascorbic acid, dehydroascorbic acid and protein-ascorbic acid found in some varieties of potatoes. Ascorbic acid was estimated by titration of a trichloroacetic-metaphosphoric acid extract; dehydroascorbic acid was determined after treatment of the extract with hydrogen sulphide; and protein-ascorbic acid after preliminary hydrolysis with 1% hydrochloric acid as outlined later.

Table III. *Effect of controlled heating on the titration values of plant tissues*

Tissue	Total ascorbic acid (mg. per 100 g.) Raw	After heating
A. Increase on heating:		
Cauliflower	19·0	31·0
Hubbard squash	3·1	4·1
Beet	2·7	6·2
String bean	1·4	2·1
Carrot	1·2	2·6
Parsnip	3·7	6·1
Red clover	26·2	42·3
B. Decrease on heating:		
Spinach	18·0	13·0
Cabbage	15·0	12·0
Onion	8·9	3·1
Broccoli	32·0	22·0
Corn	7·6	5·1
Peas	14·0	8·1
Asparagus	12·0	8·2
Turnip	35·0	18·0

Table IV. *Ascorbic acid content of some varieties of potatoes*

Variety	Ascorbic acid (mg. per 100 g.) Reduced	Dehydro	Combined	Total
A. After storage for 5 months—1935:				
Katahdin	9·2	3·5	5·0	17·7
Early Ohio	6·1	6·9	7·5	20·5
Green Mountain	7·3	6·5	7·1	20·9
Dooley	6·7	2·0	10·7	19·4
Irish Cobbler	9·3	2·3	9·1	20·7
Carmen No. 1	5·2	8·7	5·6	19·5
B. After storage for 6 weeks—1936:				
Evergreen	13·4	3·3	5·4	22·1
Eureka	16·2	3·1	3·7	23·0
Delaware	13·8	4·8	5·2	23·8
Netted Gem	13·7	4·3	3·3	21·3
Irish Cobbler	15·7	1·2	14·5	31·4

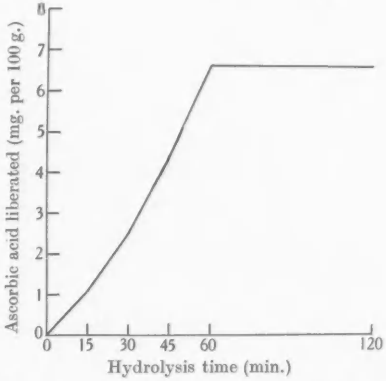

Fig. 1. Hydrolysis curve of liberation of combined ascorbic acid in 0·2 % HCl.

No clear proof has been obtained as to whether protein-ascorbic acid is a chemical compound or an adsorption complex. Fig. 1 shows a hydrolysis curve obtained by hydrolysing aliquot samples of potatoes with 0·2% hydrochloric acid for various intervals. Fresh, whole potato tissue was chopped and carefully mixed. 25 g. aliquots were immediately ground under 50 ml. 0·2% hydrochloric acid, the mixture placed in flasks filled with carbon dioxide and then tightly stoppered. The flasks were placed in the dark at 40° F. as soon as possible. The hydrolysis interval included the time taken in grinding the sample under acid. After suitable periods total ascorbic acid was determined, combined ascorbic acid being the difference between the total value after hydrolysis and that before hydrolysis found in trichloroacetic-metaphosphoric acid extracts. Since the amount of ascorbic acid liberated is logarithmically correlated with time of hydrolysis until a maximum is reached, it seems likely that actual combination does exist between protein and ascorbic acid in the original tissue.

Spectrophotometric examination

For these estimations the Hilger medium quartz spectrograph was used with an iron arc as the source of light. The exposure time was 2·5 min. in all cases. In preparing the solution of combined ascorbic acid for analysis it was found necessary to use hydrochloric acid and trichloroacetic acid in the proportions of 100 to 20. This solution was first tested alone for absorption and was found not to interfere in the region desired (Pl. II A, 1, 7; Pl. II B, 1, 6). It was then used as solvent for all determinations. The *p*H of all solutions was kept constant since this is an important factor in spectrographic estimations.

Pl. II A shows the absorption spectra of solutions of synthetic *l*-ascorbic acid of various concentrations. The position of the absorption edge was found to shift toward the longer wave-lengths with increasing concentration, the edge being at 2813 Å. for a concentration of 100 mg. per 100 ml. solution. Spectra 2, 3, 4, 5, 6, Pl. II A, are for ascorbic acid solutions of 100, 50, 25, 12 and 6 mg. per 100 ml. respectively. No. 8 is the spectrum of the iron arc alone. The number of absorption centres is proportional to the concentration ($\alpha = KC$). The maximum absorption is found at 2650–2651 Å. Dilute solutions show this band only, which is specific for reduced ascorbic acid.

Pl. II B compares the spectrum of synthetic *l*-ascorbic acid (2) with that of ascorbic acid liberated from the protein complex (4). Qualitative estimations only were made. The solution for test was made by hydrolysing a precipitate of combined ascorbic acid (precipitated from a water extract with trichloroacetic acid) with 1% hydrochloric acid, and further precipitating with trichloroacetic acid. The hydrolysed precipitate yields reduced ascorbic acid which is not precipitated by the further addition of trichloroacetic acid. By this means clear colourless solutions were obtained for analysis containing only that ascorbic acid formerly combined in the tissues and now in reduced form. Solutions thus prepared and solutions of *l*-ascorbic acid give characteristic absorption spectrophotometrically (2, 4). This absorption is removed in both cases by oxidation of the ascorbic acid present, the spectra of these solutions then being as for the solvent alone (3, 5). This indicates that the solutions contained reduced ascorbic acid, hydrochloric acid and trichloroacetic acid only, and confirms the chemical evidence that reduced ascorbic acid is hydrolysed from the protein precipitate.

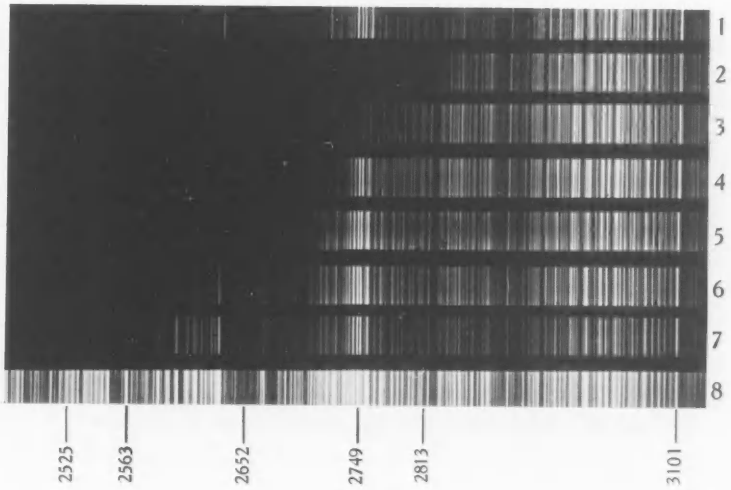

PLATE II A

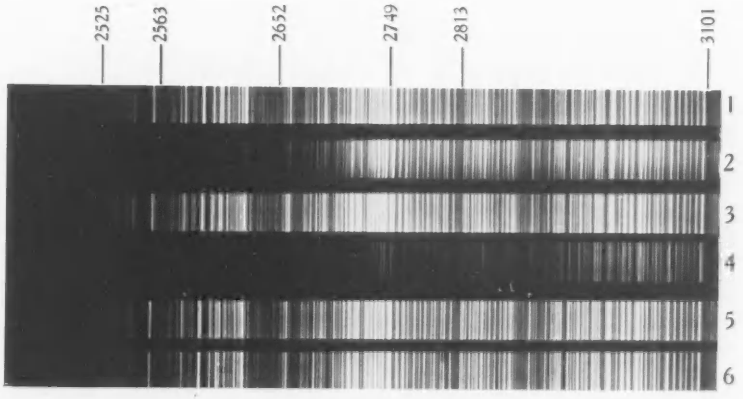

PLATE II B

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The biological assay

It has frequently been observed that young guinea-pigs maintained on a diet deficient only in vitamin C rapidly lose weight and develop marked scorbutic symptoms in 21–28 days. Many die within this time, while very few survive a 4-week period on a basal scorbutic diet or one extremely low in vitamin C. Coward & Kassner [1936] used the above observations as a basis for the quantitative assay of vitamin C, and have obtained a graduation of increase in weight in proportion to the amount of antiscorbutic material received. This method, based on the weight increase of young guinea-pigs, combined with autopsy findings and tissue analyses, was used to test the antiscorbutic properties of the protein-ascorbic acid compound.

For this purpose potato tissue was found to be a convenient source, and a precipitate consisting of protein and ascorbic acid in combination with protein was prepared from a water extract of this tissue. 2500 g. of carefully washed new potato tissue were ground into and extracted with 1 l. of cold, carbon dioxide-saturated, distilled water for 5 min. The material was then pressed through a linen filter, the liquid being centrifuged to remove suspended starch. The protein-ascorbic acid compound was precipitated from solution by means of glacial metaphosphoric acid. This precipitate was separated by centrifuging and was found to consist of protein and reduced ascorbic acid in combination with protein. A fresh quantity of precipitate was prepared daily, suspended in water and fed immediately to the test animals by means of dropping tubes. The average moisture-free weight of precipitate obtained was 10·2 g., containing the equivalent of 30 mg. of reduced ascorbic acid as found by indophenol titration after hydrolysis.

Male guinea-pigs weighing 250–300 g. were used as test animals. These animals had been on a normal diet previous to the experiment. Three groups of animals were used in the first study, receiving synthetic *l*-ascorbic acid, combined ascorbic acid and the basal diet only, respectively. The basal diet as used in this laboratory was as follows:

Bran	45%
Crushed oats	25%
Powdered skimmed milk ...	25%[1]
Yeast	3%
$CaCO_3$	1%
NaCl	1%

The animals received a fresh weighed portion of this diet daily, the same amount of food being placed before each animal. The animals were maintained in separate cages and were weighed daily. Synthetic *l*-ascorbic acid was dissolved in water to a concentration of 2 mg. per ml., and 1 ml. of a freshly prepared solution fed daily to each of 10 animals in the first group. The second group, consisting of 15 animals, received combined ascorbic acid in amounts sufficient to approximate 2 mg. of reduced ascorbic acid. The third group of 20 animals received only the basal diet. All animals were given 1 ml. of cod liver oil twice a week. The supplementary feedings were made orally by means of dropping tubes.

Table V gives a summary of the weight increases of these animals. Fig. 2 represents growth response on the basal scorbutic diet only, Fig. 3 on a diet supplemented by oral administration of 2 mg. of synthetic *l*-ascorbic acid daily and Fig. 4 on the same diet supplemented with the precipitate containing

[1] Heated in open trays under oxygen.

Table V. *Weight increases of animal groups*

Group	Supplement	Average weight of group (g.) at end of each week							Average increase in weight
		0	1	2	3	4	5	6	
I	2 mg. *l*-ascorbic acid	252·6	241·6	245·4	278·2	287·2	292·6	306·0	+54·4
II	Protein-ascorbic acid precipitate	278·4	272·8	279·6	269·6	265·2	285·0	310·8	+32·4
III	Basal diet only	234·7	222·4	219·2 (6 died)	206·9 (5 died)	188·0 (3 died)	— (1 died)	—	—

combined ascorbic acid. The curves given are representative of a larger number of animals used. The weight increases obtained with synthetic and combined ascorbic acid were not strictly comparable, possibly because of the difficulty in maintaining the level of combined ascorbic acid at 2 mg. per day. It was impossible to check chemically each feeding and it seems likely that the group on combined ascorbic acid did not receive exactly this amount, and that the precipitate varied somewhat in the amount of combined ascorbic acid contained.

All the animals receiving the basal scorbutic diet only (Group III) died within the period of the experiment. Outward symptoms of scurvy were pronounced at the end of the third week, with partial paralysis of the hind legs, ruffled hair, and swelling and signs of haemorrhage at the knee joints. Autopsy revealed marked haemorrhage, with swollen costochondral rib junctions. The animals in Groups I and II showed no outward symptoms of scurvy, and at autopsy no sign of haemorrhage was revealed in either group. Adrenal ascorbic acid analysis has been found a valuable key to the state of vitamin C nutrition in animals. Analysis of representative tissues from each group of animals resulted in low values for the animals of Group III, while the ascorbic acid content of the adrenal glands of Groups I and II was normal for this tissue.

In a further survey two groups of animals were placed on scorbutic diets. The first group of 5 guinea-pigs was allowed to remain on this diet for a period of 3 weeks, at which time freshly prepared combined ascorbic acid was given daily for an additional 3 weeks. The animals in this group had an average initial weight of 391·0 g., which fell to 343·4 g. at the end of the 3-week period, at which time the animals showed outward symptoms of scurvy. One animal died on the 25th day, autopsy revealing haemorrhages. During the second 3-week period on combined ascorbic acid the outward symptoms of scurvy disappeared, and no scorbutic symptoms could be found on autopsy.

The second group of 10 animals in this experiment received the basal diet supplemented with the protein-ascorbic acid compound which was hydrolysed and then subjected to oxidation processes specific for reduced ascorbic acid. In a period of 4 weeks frank scurvy was evident. Autopsy revealed haemorrhages typical of scurvy, the condition of the animals appearing similar to that of animals on a basal scorbutic diet only.

These experiments definitely indicate that reduced ascorbic acid in a combined form with protein was the effective agent in preventing and curing scurvy in these experimental animals. It was satisfactorily demonstrated that animals can utilize ascorbic acid in combination with protein as antiscorbutic. Since ascorbic acid is hydrolysed from the protein-ascorbic acid compound by hydrochloric acid as dilute as 0·2% it seems reasonable to conclude that hydrolysis takes place in the digestive tract to yield reduced ascorbic acid which is utilized as such.

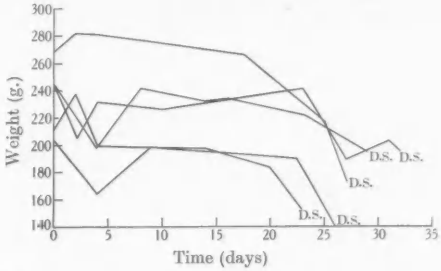

Fig. 2. Weight chart of animals receiving the basal scorbutic diet only. (D.S. = died of scurvy.)

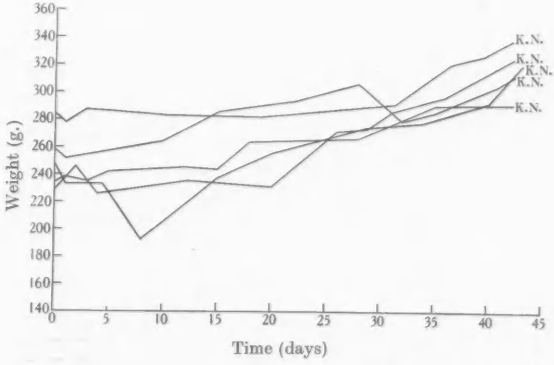

Fig. 3. Basal diet supplemented with 2 mg. synthetic *l*-ascorbic acid daily. (K.N. = killed normal.)

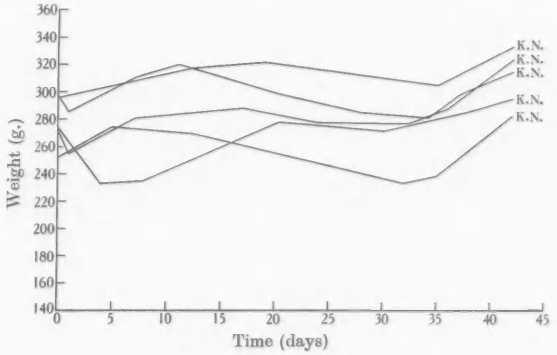

Fig. 4. Basal diet supplemented with protein-ascorbic acid compound. (K.N. = killed normal.)

Discussion

The finding that vitamin C occurs in a protein-ascorbic acid complex as well as in the form of reduced and reversibly oxidized-ascorbic acid is of importance in regard to the wide distribution and relatively high concentration of this factor in plant tissues. A number of protein complexes occur naturally, and there is evidence that other vitamins may exist in other than free form. Lovern *et al.* [1937] report that the occurrence of vitamin A is not confined to oil solutions, while Schultz [1936] states that the water-soluble factor H occurs only in a combined form with protein, and is liberated by hydrolysis. Physiologically such compounds would be more slowly absorbed in the animal body, and possibly more stable than the free vitamin.

Since protein-ascorbic acid is insoluble in trichloroacetic or metaphosphoric acid, suitable modifications should be introduced in chemical procedures using these acids for extraction. These involve preliminary hydrolysis of a weighed sample of tissue for 1 hr. with 1 % hydrochloric acid. Suitable precautions should be taken to prevent atmospheric oxidation. Reduced ascorbic acid and dehydroascorbic acid are then determined in the hydrolytic extract, the residue after hydrolysis being further extracted with trichloroacetic-metaphosphoric acid and the total ascorbic acid determined in the usual manner. The total ascorbic acid present in the tissue may be calculated from the sum of the separate titration values of the two extracts. A simple trichloroacetic-metaphosphoric acid extraction of the tissues determines reduced ascorbic acid only. Dehydroascorbic acid may be estimated by treatment with hydrogen sulphide. This method has given satisfactory results in this laboratory and was used in the analyses reported.

Summary

1. Proof of the presence of a combined form of ascorbic acid (vitamin C) in certain plant tissues has been obtained through chemical, spectrographic and biological assay. This form occurs as ascorbic acid in combination with protein, the protein-ascorbic acid compound readily yielding reduced ascorbic acid and protein on hydrolysis. Ascorbic acid in combined form has been shown to be equivalent to reduced ascorbic acid in antiscorbutic properties.

2. Combined ascorbic acid has not been found to occur in other than certain vegetable tissues. In some of these it constitutes a fairly large percentage of the total ascorbic acid present, but is not estimated by the usual chemical method of analysis since it is insoluble in the extractants used.

3. Suitable procedures are given for the estimation of combined ascorbic acid in those tissues containing this form. The results of some analyses using this method are recorded.

The authors wish to acknowledge the interest of Dr C. H. Best in this work. Spectrographic estimations were made with the aid of Dr Bateson of the MacLennan Laboratories, University of Toronto. This research was facilitated by a grant from the Banting Research Foundation, Toronto, to one of the authors (E. J. R.).

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XVII. THE HYDROLYSIS OF GLYCOGEN BY A GLYCEROL EXTRACT OF MUSCLE

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Barbour [1929] found that when glycogen is hydrolysed by a glycerol extract of skeletal muscle or of liver, the reducing activity appearing, expressed as glucose, is only about 30% of the glycogen disappearing. Case [1931] and Gray [1935] were able to confirm this observation, but Carruthers [1935] did not obtain consistent results. From the products of hydrolysis Barbour isolated, by a relatively simple method, a substance which he identified as a trisaccharide. Carruthers & Lee [1935] and Gray were, however, unable to repeat this isolation, and Carruthers [1935] brought forward evidence which he believed to indicate that a mixture of maltose and dextrin, and not a trisaccharide, results from the hydrolysis of glycogen by a glycerol extract of muscle or liver tissues. Winter [1937] finds that the end product of the action of a glycerol extract of heart muscle on glycogen is glucose, although he believes that a trisaccharide, rather than a disaccharide, may be formed intermediately.

The present investigation was undertaken to confirm, if possible, the statement that a trisaccharide is formed by the hydrolysis of glycogen under the conditions described by Barbour, and to examine the chemical nature of the purified material.

Methods

Glycogen was prepared from rabbit liver and from *Mytilus edulis* by Pflüger's method. All the specimens used had $[\alpha]_D$ close to $+195^\circ$ and ash content about 0·25%. Glycogen was estimated by the method of Evans *et al.* [1931], sugar being determined by the method (Reagent 50) of Shaffer & Somogyi [1933].

A glycerol extract of rabbit muscle was prepared by the method of Hunter & Dauphinee [1924].

Results

Hydrolysis of glycogen by a glycerol extract of muscle

In Table I are given typical results for the hydrolysis of 100 ml. of a 3·5% solution of glycogen in phosphate buffer at *p*H 6·4 by 25 ml. of a glycerol extract of rabbit skeletal muscle. The hydrolysis was carried out in the presence of toluene at 37°. The ratio (reducing power appearing)/(glycogen disappearing) is somewhat greater than the value (1/3) which might be expected if a trisaccharide were formed, although the difference is not large. The final reducing power in a large number of hydrolyses has been very close to 38% of the amount of glycogen disappearing. These results might be considered to indicate that a trisaccharide is formed by the hydrolysis of glycogen by a glycerol extract of muscle. Nevertheless, it seemed possible that the primary product of the hydrolysis might be glucose or maltose, and that a trisaccharide had been formed

[1] Work carried out during the tenure of a Carnegie Research Scholarship.

Table I

Time hr.	Glycogen g.	Glycogen destroyed g.	Reducing power. Glucose equivalent g.	Increase in reducing power g.	$\frac{\text{Increase in reducing power}}{\text{Glycogen disappearing}} + 100$
0	3·38	0	0·05	0	—
24	1·82	1·56	0·56	0·51	33
36	1·67	1·71	0·71	0·66	39
48	1·00	2·38	0·96	0·91	38

secondarily under the influence of the glycerol or of the enzyme. Accordingly the control experiments shown in Table II were carried out. In each case a suitable concentration of the sugar in phosphate buffer at *p*H 6·4 was mixed with 25% of its volume of glycerol or glycerol extract of rabbit muscle, and

Table II

Sugar	Enzyme	Temp. °C.	Time hr.
Glucose	Absent	37	30
	Present	37	48
Maltose	Absent	37	44
	Present	37	48
Glucose + maltose	Absent	37	48
	Present	37	36
Glycogen	Absent	37	38
	Absent	100	1

incubated at 37° in the presence of toluene, or heated to 100°. In no case was any significant change of rotatory power or reducing activity found in these control experiments, and it seemed probable that the figures indicating the possible production of a trisaccharide by the hydrolysis of glycogen, were of primary importance.

Attempts to isolate the products of hydrolysis

Barbour states "the sugar itself was...isolated by precipitation as a barium compound on the addition of excess barium hydroxide from a solution in 70% alcohol. This compound was dissolved in water, decomposed by a current of CO_2, the last trace of barium removed with sulphuric acid, and the filtrate evaporated to dryness. The sugar was purified by a repetition of this procedure". We have repeatedly attempted to obtain a purified sugar by this simple process but in every case the product has contained a very high proportion of ash. The addition of barium hydroxide to an aqueous solution of the products of hydrolysis results in a precipitate which appears to consist almost entirely of barium phosphate. Accordingly this precipitate has been discarded in many cases, the carbohydrate material being subsequently precipitated from solution in the presence of excess barium hydroxide by the addition of alcohol to make 70%. Repetition of this process, followed by evaporation at low temperature, yielded a syrup which could be dried to an amorphous white powder containing about 10% of ash and having a reducing power about 14% that of glucose. The yield was approximately 5% of the glycogen hydrolysed. When pure maltose, dissolved in phosphate buffer, was subjected to the above process of isolation it acquired about 15% of ash, and it seemed unlikely that this process could be expected to yield an ash-free material from the products

of hydrolysis of glycogen. Numerous unsuccessful attempts to diminish the ash content of the products of glycogen hydrolysis were made by fractional precipitation with methyl and ethyl alcohols at different *p*H, and with alcohol-ether mixtures. Finally a modification of the copper-baryta precipitation method of Fleury & Ambert [1929] was applied. This process, which gave a 70% recovery of carbohydrate material, yielded a product having an ash content of 4·8%; repetition of the process failed to reduce the ash content further. Acetylation of the product with acetic anhydride in pyridine, followed by purification of the acetylated material, gave an acetyl derivative with an ash content of 1·6%. The original product and the acetyl derivative were both analysed, the results being calculated on an ash-free basis.

Properties of the unacetylated product

The product was an amorphous white, slightly hygroscopic powder, easily soluble in water but insoluble in organic solvents. Its reducing power was 14% of that of glucose, and its $[\alpha]_D$ was $+171°$. It yielded a phenylosazone of a form similar to that described by Barbour—small star-shaped aggregates of needles—but attempts to recrystallize this derivative were not successful. The original product was rapidly hydrolysed on heating with 2·4% HCl at 100°. Glucose appeared to be the main hydrolytic product and was identified by the crystalline form of the osazone and its melting point when mixed with an authentic specimen of glucosazone. The course of acid hydrolysis was followed polarimetrically and by reducing activity, the results being plotted in Fig. 1. The hydrolysis is practically complete in 90 min. under these conditions.

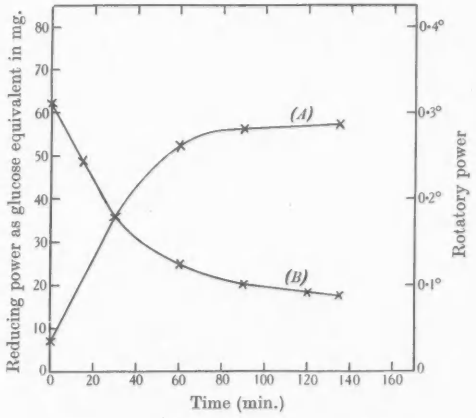

Fig. 1. Hydrolysis curve for 50·8 mg. of product dissolved in 26·5 ml. of 2·4% HCl and heated at 100°. Course of hydrolysis followed by reducing power (curve *A*) and by rotatory power (curve *B*).

Elementary analysis of the original product gave C=41·50%, H=6·81% (calculated for a trisaccharide—C=42·86%, H=6·35%). Ash=4·8%, containing only a trace of phosphorus.

Properties of the acetyl derivative

The product of acetylation was an amorphous white powder, insoluble in water, but soluble in the usual organic solvents. In chloroform solution $[\alpha]_D = +92°$. Elementary analysis gave C = 49·23 %, H = 5·75 % (calculated for a trisaccharide having 11 hydroxyl groups acetylated—C = 49·70 %, H = 5·63 %). Ash content = 1·6 %. Acetyl content = 48·6 % (calculated for a trisaccharide having 11 hydroxyl groups acetylated—48·97 %); mol. wt. (Rast) = 1010 (calculated for a fully acetylated trisaccharide—966).

Discussion

Barbour [1929] considers that the glycerol extract of muscle he used contains a glycogenase whose action on glycogen yields a trisaccharide as the sole product of hydrolysis. Carruthers [1935] brought forward evidence to show that a glycerol extract of muscle contains both diastase and maltase, although the action of the maltase is partly inhibited in the presence of glycerol. This author inclines to the view that such an extract hydrolyses glycogen to a mixture of maltose and non-reducing dextrin, although the possibility that a small amount of trisaccharide is formed could not be completely ruled out. Although we have obtained more consistent results with the hydrolysis of glycogen than did Carruthers [1935] [cf. Case, 1931; and Gray, 1935], nevertheless the reducing power appearing has always been more than 30 % of the glycogen disappearing, the average figure being about 38 %. Carruthers's belief that his results more probably indicate the formation of a mixture of maltose and dextrin than that of a trisaccharide is based more on an investigation of the nature of the enzyme system concerned than on examination and purification of the product prepared according to Barbour's directions. The majority of the experiments of Carruthers, and of Carruthers & Lee, were carried out with a dialysed, glycerol-free enzyme preparation. As these authors indicate, such experiments cannot decide whether or not a trisaccharide can be isolated under the conditions of Barbour's experiments. Evidence bearing on this question must come from an attempted repetition of Barbour's investigations.

Barbour does not say whether or not his preparations contained ash, but it seems, from our results, that he could hardly have obtained an ash-free product by the simple method of barium precipitation he describes for the isolation of the free sugar. Carruthers & Lee found that "attempts to purify fractions by precipitation with barium hydroxide were unsuccessful". The high ash content has been a most troublesome feature of our preparations, and although our analytical figures have been corrected for ash, the high ash content of the preparation necessarily introduces an uncertainty into the interpretation of any analytical results.

As well as isolating a trisaccharide Barbour obtained a compound of lower reducing power which he believed to be an anhydrotrisaccharide, formed from the trisaccharide by the removal of one molecule of water. This compound had a reducing power only 8·5 % of that of glucose, compared with 31 % for the trisaccharide, but otherwise its properties were not very different from those of the latter compound. The product obtained in the present investigation appears to resemble Barbour's anhydrotrisaccharide rather than his trisaccharide, as judged by the comparatively low reducing power of our preparation (14 %).

Pringsheim [1924] isolated a substance which he called "amylotriose" by the hydrolysis of glycogen or amylopectin with cold concentrated HCl. Lohmann

[1926], investigating the diastatic hydrolysis of glycogen, isolated a sugar which he suggested might be identical with Pringsheim's amylotriose. Barbour believes, on the basis of difference in rotation and reduction, that his trisaccharide is different from that of Pringsheim or of Lohmann. The properties of the various presumed trisaccharides formed from glycogen are summarized in Table III, the properties of the corresponding acetyl derivatives being given in

Table III

Author	$[\alpha]_D$	Reducing power (glucose = 100)	Mol. wt. (theory = 504)	% carbon (theory = 42·86)	% hydrogen (theory = 6·35)
Pringsheim	+124°	26	513	42·72	6·47
Lohmann	137·5	9·7	480	—	—
Barbour	154	31	505	42·85	6·34
Present authors	171	14	—	41·50	6·81
Barbour (anhydro-trisaccharide)	158*	8·5	481† (theory = 486)	—	—

* Calculated from the $[\alpha]_{Hg}$ of 187° given by Barbour. As Carruthers & Lee point out, the data given by Barbour establishes the $[\alpha]_{Hg}$ as 374°, and not 187°, the figure Barbour gives.

† As Carruthers & Lee point out, the data given by Barbour establishes the mol. wt. as 240 and not 481.

Table IV

Author	$[\alpha]_D^{CHCl_3}$	Mol. wt. (theory = 966)	Acetyl content (theory = 48·97%)	% carbon (theory = 49·67)	% hydrogen (theory = 5·63)
Pringsheim	+125°	1020	47·41	—	—
Lohmann	127	1010	46·20	48·78	5·72
Present authors	92	1010	48·60	49·23	5·75
Barbour (anhydro-trisaccharide)	—	961 (theory = 906)	47·5 (theory = 47·5)	—	—

Table IV. It seems unlikely from the figures collected here that any one of these products can be identical with any other, and adequate evidence that any of them is a single substance is lacking. The results obtained by the present authors for the acetyl derivative are reasonably close to the theoretical figures for an undeca-acetyl trisaccharide, but it would be unwise to suggest, on this basis alone, that the isolated material consists of a trisaccharide. The data believed by the various authors to characterize a trisaccharide might easily be obtained from a mixture of glucose, maltose and reducing dextrin or oligosaccharide [cf. Carruthers, 1935]. The preparation of an osazone having a characteristic crystalline appearance and a nitrogen content similar to that required for the osazone of a trisaccharide has often been cited as evidence for the existence of a trisaccharide. The doubtful value of such evidence is clear from a recent review by Ling [1937] of the nature of certain hydrolytic products from starch. Ling concludes that many of the supposed substances intermediate between starch and maltose, including a presumed trisaccharide, are co-ordination compounds of maltose and non-reducing dextrin. Particularly instructive is the case of the so-called "*iso*maltose" for which a characteristic osazone has frequently been described. Ling points out that it is practically impossible to separate "*iso*-maltose" into its constituents by fractionation with alcohol; nevertheless it appears to be a mixture of substances, and the appearance of "*iso*maltosazone" can be simulated by recrystallizing maltosazone in the presence of dextrin. It

is clear that the nitrogen content of an "osazone" of this type might easily approach that for the osazone of a trisaccharide.

Until a purified trisaccharide with well-characterized crystalline derivatives can be obtained in amount sufficient to permit an extensive examination, the question of whether or not a trisaccharide can be formed by the hydrolysis of glycogen should remain open. The evidence available certainly does not warrant the assumption that a definitive trisaccharide ever constitutes the major portion of the products of glycogen hydrolysis.

Experimental

Purification of crude product from glycogen hydrolysis [cf. Fleury & Ambert, 1929]. 1 g. of the product obtained according to Barbour's directions was dissolved in 10 ml. of water and treated with small amounts of 20% $CuSO_4$ and $N/3{\cdot}6$ $Ba(OH)_2$ solutions alternately until total volumes of 21·5 and 240 ml. respectively had been added. The mixture was stirred at 0° for 1 hr. and the precipitate centrifuged off and suspended in water. 50% H_2SO_4 was added until just acid to Congo red, and the precipitated $BaSO_4$ centrifuged off and discarded. The copper in the filtrate was removed by H_2S and, after aeration, the solution was freed from Ba^{++} ions and SO_4^{--} ions by the addition of appropriate amounts of $Ba(OH)_2$ and H_2SO_4. The solution was concentrated to a syrup under reduced pressure and dried with alcohol to an amorphous powder. Yield = 0·7 g. i.e. 70%.

Analysis of the product.

4·195 mg. gave 6·080 mg. CO_2 and 2·450 mg. H_2O. Res. = 0·202 mg. (Weiler), whence C = 41·50% and H = 6·81%. Ash = 4·8%.

22·84 mg. in 25 ml. H_2O gave $\alpha = +0{\cdot}148°$; $l = 1$, whence $[\alpha]_D = +170{\cdot}6°$.

22·84 mg. in 25 ml. H_2O estimated as 3·03 mg. glucose by Shaffer-Somogyi method. Reducing power is therefore 13·9% that of glucose.

Hydrolysis curves. 50·80 mg. of product were dissolved in 25 ml. of H_2O and 1·5 ml. of concentrated HCl added. The course of hydrolysis on heating to 100° was followed polarimetrically [cf. Young, 1937] and by the estimation of reducing power.

Time min.	Rotation °	Reducing power mg. equiv. glucose
0	+0·312 ($[\alpha]_D^{HCl} = +171°$)	7·2
15	0·243	—
30	0·177	35·8
60	0·124	52·4
90	0·102	56·0
120	0·093	—
135	0·085	56·9
Theoretical	0·102	51·8

Acetylation. 150 mg. of product were heated with 0·9 ml. of pyridine and 0·6 ml. of acetic anhydride to 50° for 2 hr., and left overnight at room temperature. The mixture was filtered into ice-cold water and the precipitate spun off, dissolved in dry chloroform and evaporated to dryness after moisture had been removed by standing over anhydrous Na_2SO_4. The residue was dissolved in hot, absolute alcohol. On cooling, the solution deposited an amorphous white powder which was centrifuged and dried *in vacuo*. Yield = 23 mg., i.e. 12% of theoretical.

Analysis of acetyl derivative.

3·560 mg. gave 6·320 mg. CO_2 and 1·810 mg. H_2O. Res. = 0·06 mg. (Weiler), whence C = 49·23% and H = 5·75%. Ash = 1·6%.

0·646 mg. in 6·028 mg. camphor depressed melting-point by 4·2° (Weiler); mol. wt. = 1010.

10·303 mg. liberated equiv. 11·45 ml. $N/100$ NaOH on complete hydrolysis. Acetyl content = 48·6% $O.CH_3$.

14·58 mg. in 10 ml. $CHCl_3$ gave $\alpha = +0{\cdot}067°$; $l = 0{\cdot}5$ whence $[\alpha]_D$ $CHCl_3 = +91{\cdot}9°$.

SUMMARY

1. Barbour's statement that when glycogen is hydrolysed by a glycerol extract of rabbit muscle the reducing power appearing, calculated as glucose, is only about one-third of the amount of glycogen disappearing, has not been entirely confirmed, in that the reducing power appearing in our experiments was about 38% of the glycogen disappearing.

2. Attempts to prepare a purified trisaccharide according to Barbour's directions invariably yielded a product containing a large amount of ash. However, an acetyl derivative was prepared whose analytical results were close to those for an undeca-acetyl trisaccharide. The possibility that the analytical figures obtained might be given by a mixture of maltose and oligosaccharide or dextrin is stressed. The relationship of the results of the present investigation to those of Pringsheim, Lohmann and Barbour is discussed.

3. The conclusion is drawn that until a purified trisaccharide with well-characterized crystalline derivatives can be prepared from glycogen in amount sufficient to permit extensive examination, the question of whether or not a trisaccharide can be formed by the hydrolysis of glycogen should remain open.

We wish to express our thanks to Prof. J. C. Drummond for his advice and criticism.

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XVIII. PHOSPHORYLATION BY THE LIVING BACTERIAL CELL[1]

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Since the classical work of Harden & Young [1905] an immense amount of evidence has been brought forward favouring the intimate association of glucolysis and phosphorylation. The greater part of the investigations, however, has been carried out with unnatural systems, such as cell-free juices, acetone or toluene treated cells, or poisoned systems.

Unquestionably phosphorylation is related to the dissimilation of glucose under the unnatural conditions. The question arises, however, as to whether phosphorylation is intimately associated with glucolysis in the living cell. Macfarlane [1936] established a relationship in yeast between a decrease in orthophosphate and rapid fermentation by comparing orthophosphate in the trichloroacetic acid extracts of yeast cells fermenting glucose and not fermenting glucose. In addition to the orthophosphate, the following fractions were determined:

Labile phosphate, calculated from Lohmann's equation,

$$\text{"Labile" } P = \Delta_{7 \text{ min.}} - {}_{0 \text{ min.}} - \Delta_{30 \text{ min.}} - {}_{7 \text{ min.}};$$

total acid-soluble phosphorus, determinable after ashing with concentrated sulphuric acid and hydrogen peroxide and organic phosphorus fraction, calculated as the total acid-soluble minus the sum of the orthophosphate and the labile phosphorus. In these experiments the decrease in orthophosphate was not correlated with an increase in organic phosphate, but it has since been shown [Macfarlane, 1937] that phosphoric esters, mainly hexosediphosphate, are present in increased amount during fermentation.

In the present communication the living bacterial cell has been studied. The phosphoric esters in the trichloroacetic acid extract of the cells were classified according to ease of hydrolysis in *N* HCl at 100°. The esters from the extract of cells fermenting glucose were quantitatively compared with those from cells not fermenting glucose. Under the conditions of our experiments there exists a correlation between a decrease in readily determinable phosphorus and an increase in esterified phosphorus occurring on addition of glucose.

Experimental

Methods

Aerobacter aerogenes was grown in a medium of 50 g. of peptone, 50 g. of glucose, 125 ml. of 0·5 *M* potassium phosphate buffer (*p*H 7·0) and 10 l. of water. Cultures which were never over 12 hr. old were centrifuged and the cells suspended in sterile water in such a concentration that a 10 or 12 ml. sample of the

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suspension was approximately equivalent to 1 g. (wet wt.) of bacterial cells. The suspension was equally divided between two flasks with 50 ml. in each. 10 or 12 ml. samples were taken before and at various intervals after the addition of 2 ml. of a solution of glucose to one flask, and the addition of 2 ml. of water to the other. To the samples were added 5 ml. of a 25% solution of trichloroacetic acid. After storing overnight in the ice-box the samples were centrifuged and the supernatant liquid was analysed for orthophosphate which was determinable, (*a*) readily, (*b*) after hydrolysis in *N* HCl at 100° for 7, 12, 30, 120 and 300 min. respectively, and (*c*) after ashing with sulphuric acid and hydrogen peroxide.

Phosphorus was determined by Briggs's [1922] modification of the Bell-Doisy phosphate method in Exps. 6, 9 and 14, and by the method of Küttner & Lichtenstein [1930] in Exps. 4 and 5. The values obtained were plotted against time of treatment in *N* HCl at 100° (Fig. 1).

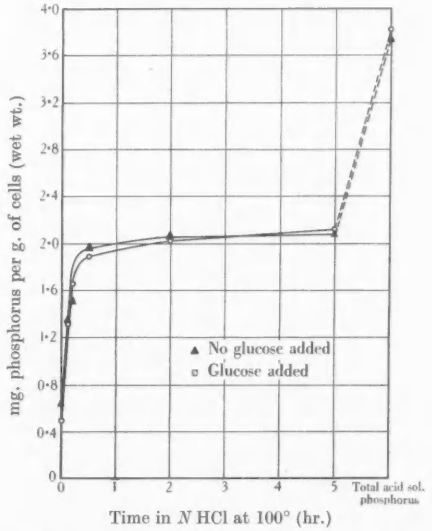

Fig. 1. Hydrolysis curves of phosphoric acid esters present in the trichloroacetic acid extract of bacterial cells. Broken lines are projected to total acid soluble phosphorus values obtained on ashing. (Exp. 14*b*, Table I.)

It is seen that phosphoric acid esters are present which are completely hydrolysed in 30 min. and that the amount of these easily hydrolysable esters present may be calculated from the equation:

Easily hydrolysable P = P determinable after 30 min. hydrolysis − readily determinable P.

The difficultly hydrolysable phosphoric acid esters may be calculated from the equation:

Difficultly hydrolysable P = total acid soluble P − phosphorus determinable after 30 min. hydrolysis.

The above curves have been presented to show the types of phosphoric acid compounds present, as well as their quantitative relationship to fermentation.

In Table I are given quantities of esters present in the trichloroacetic acid extract of bacterial cells fermenting and not fermenting glucose.

Table I. *Phosphorus fractions present in the trichloroacetic acid extracts of living cells of* Aerobacter aerogenes[1]

Quantities in mg. phosphorus per g. of cells (wet wt.)

		(A) No glucose added				(B) Glucose added			
Exp.	Time after addition of glucose to B min.	Readily determinable P	Easily hydrolysable P 30–0	Difficultly hydrolysable P *T*–30	Total acid soluble P	Readily determinable P	Easily hydrolysable P 30–0	Difficultly hydrolysable P *T*–30	Total acid soluble P
14 *a*	0	0·65	1·35	1·78	3·78	0·58	1·52	1·76	3·86
b	3	0·64	1·32	1·78	3·74	0·48	1·42	1·92	3·82
c	15	0·58	1·45	1·74	3·77	0·53	1·33	1·88	3·74
d	420	0·79	0·91	1·17	2·87	0·64	1·07	1·53	3·24
9 *a*	0	0·67	1·47	1·61	3·75	0·65	1·57	1·67	3·89
b	10	0·67	1·51	1·44	3·62	0·45	1·64	1·90	3·99
c	30	0·70	1·53	1·55	3·78	0·50	1·55	1·81	3·86
d	420	0·89	0·57	1·10	2·56	0·70	0·77	1·17	2·64
6 *a*	0	0·54	0·44	0·66	1·64	0·45	0·55	0·67	1·67
b	40	0·51	0·71	1·06	2·28	0·28	0·79	0·97	2·04
c	330	0·47	0·94	0·80	2·21	0·40	0·80	1·42	2·62
5 *a*	0	0·42	0·76	0·86	2·04	0·52	0·77	0·89	2·18
b	25	0·47	1·06	0·75	2·28	0·33	1·06	1·00	2·39
c	720	0·71	1·00	0·96	2·67	0·55	0·89	1·04	2·48
4 *a*	5	0·59	1·05	0·94	2·58	0·35	1·17	1·60	3·12
b	30	0·56	0·96	1·18	2·70	0·25	1·27	1·19	2·71
c	180	0·58	1·12	1·04	2·74	0·37	1·16	1·21	2·74

* *A. indologenes* instead of *A. aerogenes* was used in No. 4.

It is apparent that the readily determinable phosphorus is less and the esterified phosphorus is greater in the presence of glucose than in its absence. Following the addition of glucose to B (Table I), there occurs a decrease in readily determinable phosphorus, followed by a gradual rise as the latter stages of the fermentation are reached. Often it is the difficultly hydrolysable phosphorus which has increased in the presence of glucose but frequently the easily hydrolysable phosphorus is increased. The variation in the total acid-soluble phosphorus of the same suspension requires some explanation. The total phosphorus of the acid extract depends in part on the time the cells are left in contact with the trichloroacetic acid. Since the time of extraction varied with the samples taken at different times, the total phosphorus values show some variation. It should, perhaps, be noted in this connexion, that the time of extraction for any set of samples (A and B) at a given time was always the same. In Exp. 6 we were unable to correlate an increase in esterified phosphate with the decrease in orthophosphate at the 40 min. period. This result may possibly have been due to an error in determining the total phosphorus since in only one other determination (Exp. 5*c*) is the total acid soluble phosphorus less in the presence of glucose than in its absence. The decrease of orthophosphate with the increase of esterified phosphorus shown by Table I is a fundamental characteristic of phosphorylation.

Estimation of the number of living bacterial cells

To determine the number of living bacterial cells present, a comparison was made between microscopic and plate counts. The former was obtained by use of the Petroff-Hauser bacteria counting cell. Plate counts were determined on the following medium: infusion from 5 g. calf liver, 3 g. of peptone (Difco), 10 g. of glucose, 25 ml. 0·8 *M* potassium phosphate buffer (*p*H 7·0), 20 g. of agar and 1000 ml. of water.

Table II. *Comparison of microscopic counts and plate counts of an 11 hr. culture of* Aerobacter aerogenes

Exp. 1				Exp. 2			
Theoretical count*	No. of plates	Plate count (Av.)	% viable cells	Theoretical count*	No. of plates	Plate count (Av.)	% viable cells
956	1	774	80·96	1012	1	967	95·55
478	3	476·7	99·72	506	3	459·6	90·83
239	3	217·6	91·05	253	2	249·5	98·62
119·5	3	93·3	78·08	126·5	3	109·7	86·72
47·8	1	42	87·86				
		Av.	88·67			Av.	91·71

Av. of 1 + 2 = 90·01 %.

* Calculated from the microscopic count.

Results are given in Table II. It is apparent that 90 % of the cells were capable of proliferation. Probably the percentage of living cells is higher, since some cells may be alive but not capable of proliferation.

Behaviour of cells from a 15-day culture with respect to phosphorylation

Since a small percentage of dead cells are probably present even in a 12-hr. culture of *A. aerogenes*, it is of interest to know the behaviour of a suspension of cells from an old culture with respect to phosphorylation. Exp. 7, Table III,

Table III. *The effect of the addition of glucose on phosphorus distribution of a suspension of cells from a 15-day culture of* Aerobacter aerogenes

Quantities in mg. phosphorus per g. of cells (wet wt.)

		(A) No glucose added				(B) Glucose added			
Exp.	Time after addition of glucose to B min.	Readily determinable P	Easily hydrolysable P 30–0	Difficultly hydrolysable P *T*–30	Total acid soluble P	Readily determinable P	Easily hydrolysable P 30–0	Difficultly hydrolysable P *T*–30	Total acid soluble P
7	0	0·26	0·59	1·18	2·03	0·27	0·53	1·07	1·87
	25	0·29	0·51	1·08	1·88	0·29	0·42	1·04	1·75
	300	0·29	0·37	0·98	1·64	0·30	0·35	0·92	1·57

was carried out in the same manner as the experiments described above with the exception of the use of a 15-day in place of a 12-hr. culture. The readily determinable phosphorus does not decrease in the presence of glucose, nor does the esterified phosphorus increase.

Increase of the readily determinable phosphorus with time

It has been noted that in a suspension of bacterial cells, the readily determinable phosphorus increases with time. We have considered the possibility of this phenomenon being associated with the depletion of the carbohydrate reserve of the cell.

It seems possible that when the bacterial cell has used up the free glucose, it may then use the carbohydrate reserve of the cell as a source of energy. We may expect some phosphorus to be continuously esterified with the reserve as it is used, thus remaining in organic combination; but as the amount of reserve decreases, the quantity of unesterified phosphorus will rise, just as it does at the end of the glucose fermentation.

Such an explanation may be tested by seeing whether the reserve is phosphorylated, for, in this case, the change in the distribution of phosphorus occurring on addition of glucose should be more apparent with cells in which the reserve has been depleted, just as would be the case on adding glucose to cells with no glucose at all, as compared with cells with a small amount of glucose present. In order to test this point, 10 ml. of the bacterial suspension after 48 hr. vigorous aeration were placed in each of two centrifuge cups. 2 ml. of 12% glucose solution were added to one cup and to the other 2 ml. of water. As soon as fermentation commenced, as evidenced by the evolution of CO_2, 5 ml. of trichloroacetic acid were added to each tube. Within 48 hr. the cells were centrifuged and the supernatant liquid was analysed as in the experiments described above. Results are shown in Table IV. It is interesting to note:

Table IV. *Effect of aeration on the change of phosphorus distribution occurring on addition of glucose to* Aerobacter aerogenes

Quantities in mg. phosphorus per g. of cells (wet. wt.)

		Before aeration*				After 48 hr. aeration			
Exp.		Ortho-P	Easily hydrolysable P 30–0	Difficultly hydrolysable P *T*–30	Total acid soluble P	Ortho-P	Easily hydrolysable P 30–0	Difficultly hydrolysable P *T*–30	Total acid soluble P
4	Before addition of glucose	0·59	1·05	0·94	2·58	1·87	0·47	0·49	2·83
	After addition of glucose (30 min.)	0·25	1·27	1·19	2·71	1·38	0·76	0·76	2·90
	Difference due to addition of glucose	−0·34	+0·22	+0·25	+0·13	−0·49	+0·29	+0·27	+0·07
	Change due to aeration	—	—	—	—	−0·15	+0·07	+0·02	−0·06
5	Before addition of glucose	0·52	0·77	0·89	2·18	1·51	0·45	0·62	2·58
	After addition of glucose (25 min.)	0·33	1·06	1·0	2·39	1·19	0·47	1·34	3·00
	Difference due to addition of glucose	−0·19	+0·29	+0·11	+0·21	−0·32	+0·02	+0·72	+0·42
	Change due to aeration	—	—	—	—	+0·13	−0·27	+0·61	+0·21

* From Exps. 4 and 5, Table I; other portions of the same suspensions were used in the aeration experiments.

(1) that on aeration the esterified phosphorus is broken down with a consequent increase in orthophosphate; (2) that on addition of glucose to both suspensions of aerated and unaerated cells, significant changes in phosphorus distribution occur; and (3) that the changes occurring are greater in the case of the aerated

cells than in the non-aerated cells. The last point indicates that the reserve is phosphorylated, and hence supports the belief that a great part, at least of the increase of orthophosphate with time, is due to the depletion of reserve substances in the bacterial cell.[1]

Discussion

In showing that phosphorylation is associated with glucolysis in the living bacterial cells, a certain additional value is placed on the past research with non-living systems.

Considerable evidence has been brought forward favouring the importance of phosphorylation in bacterial metabolism. Virtanen & Tikka [1930] have isolated a disaccharide phosphoric ester from *Lactobacillus casei*, and Tikka [1935] has investigated the fermentation of phosphoglyceric acid, α-glycerophosphoric acid and hexosediphosphate. Neuberg & Kobel [1933] have shown that phosphoglyceric acid yields pyruvic acid and phosphoric acid in the presence of *L. delbrückii*. Werkman *et al.* [1936] were the first to isolate phosphoglyceric acid from bacteria, and Stone & Werkman [1937] have shown the rather general occurrence of this ester among members of the order Eubacteriales.

Evidence has been brought forward by Werkman *et al.* [1937] that certain bacteria which form phosphoglyceric acid can dissimilate glucose in the presence of a concentration of sodium fluoride which inhibits the dissimilation of the principal phosphoric acid esters of the Embden-Meyerhof scheme. They suggested that two mechanisms occur; one involving phosphoglyceric acid and the other not.

The objection might be raised that the phosphoric esters whose breakdown is inhibited by the concentration of sodium fluoride used constitute only stabilization products of the active forms. Evidence for this objection is lacking, however, and these experiments must be interpreted as being evidence against the Embden-Meyerhof scheme as the only route of glucose dissimilation by bacteria. Such a conclusion, of course, does not rule out the role of phosphorylation or the Embden-Meyerhof scheme.

Summary

In comparing the quantities of phosphoric acid esters present in the trichloroacetic acid extracts of living bacterial cells fermenting glucose to the quantities present in the extract of cells not fermenting glucose, it has been found that:

(1) The readily determinable or unesterified orthophosphate is less in the presence of glucose than in its absence.

(2) The esterified phosphorus is greater in the presence of glucose than in its absence.

(3) These changes in the distribution of phosphorus occurring on addition of glucose are fundamental characteristics of phosphorylation, hence evidence has been given for the occurrence of phosphorylation in the living bacterial cell and its association with glucolysis.

(4) An explanation is offered for the increase of the orthophosphate with time in a suspension of cells.

[1] A certain amount of this increase may be due to autolysis, this process possibly freeing phosphorus from its various compounds.

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XIX. MICRO-DETERMINATION OF α-KETOGLUTARIC ACID

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α-Ketoglutaric acid is an intermediate metabolite in the oxidative breakdown of carbohydrate and of various amino-acids and its quantitative determination is therefore a matter of considerable interest. The method used in previous work for the identification and determination was the isolation of the 2:4-dinitrophenylhydrazone [Krebs, 1933; Weil-Malherbe & Krebs, 1935; Simola, 1936; Martius & Knoop, 1937]. This method is satisfactory if the identification is the main object, but it is not suitable for the quantitative determination of smaller quantities of α-ketoglutaric acid, where the solubility of the 2:4-dinitrophenylhydrazone (0·26 % according to Martius [1937]) is sufficiently great to give rise to low yields.

The method described in this paper is accurate, specific and applicable to quantities from 0·2 mg. upwards. The principle is as follows: α-ketoglutaric acid is first converted into its 2:4-dinitrophenylhydrazone. This is extracted with ether from the aqueous solution. The hydrazone is then freed from ether, dissolved in alkali and treated with permanganate in acid solution. Permanganate oxidizes the hydrazone quantitatively to succinic acid. The latter is determined manometrically by means of succinic dehydrogenase.

Oxidation of α-ketoglutaric 2:4-dinitrophenylhydrazone

The reaction on which the new method is based is the quantitative conversion of α-ketoglutaric 2:4-dinitrophenylhydrazone into succinic acid. I found that this reaction is brought about by oxidation with permanganate in acid medium. The best yields were obtained if the oxidation was carried out at room temperature in 5 % H_2SO_4. In the presence of an excess of $KMnO_4$, 7 mols. CO_2 and 1 mol. succinic acid are liberated from each mol. of α-ketoglutaric 2:4-dinitrophenylhydrazone. For instance, 0·99 mg. hydrazone gave 483 μl. CO_2 in a manometric experiment (calc. for 7 CO_2 : 480 μl.). At 20° more than 90 % of the CO_2 was evolved within 15 min. Table I shows that the theoretical amount of succinate is obtained under these conditions.

As will be seen, the results were satisfactory if 2 mg. or more of the hydrazone were used. With these quantities the error did not exceed 4·5 %. The percentage error naturally becomes greater when the quantities are smaller. Considerable variation of the concentration of the acid did not greatly affect the yield of succinic acid. In the presence of 20 % H_2SO_4 the yield was about 90 % and with 5 or 20 % acetic acid about 85–90 %. Incomplete yields were obtained when very concentrated solutions of the hydrazone were acidified and treated with permanganate. 1 ml. of the acidified solution should not contain more than 1 mg. hydrazone.

Temperature has no appreciable effect on the yields of succinic acid between 20 and 100°, provided that prolonged heating with acid permanganate is avoided.

Table I. *Oxidation of pure α-ketoglutaric 2:4-dinitrophenylhydrazone with $KMnO_4$*

A known amount of the hydrazone, dissolved in 10 ml. 0·01 *M* Na_2CO_3, was placed in a Kutscher-Steudel extractor. One-tenth vol. 50% H_2SO_4 and 3 ml. 2% $KMnO_4$ were added in succession. After 15 min. the mixture was extracted with ether. Succinic acid was determined as described previously [Krebs, 1937].

	Succinic acid determination		
mg. hydrazone used	μl. O_2 measured	μl. O_2 calculated	% recovery
10·58	366	364	99·4
10·58	367	364	100·5
5·29	181	182	99·4
5·29	178	182	97·8
3·82	127	131	97·0
3·82	126	131	96·3
3·82	129	131	98·5
1·98	70	68	102·9
1·98	69·5	68	102·2
0·85	31·9	29·2	109·0
0·85	29·1	29·2	100·0
0·425	12·0	14·6	82·0
0·425	17·8	14·6	122·0

Experimental details of the determination

Reagents required:

(1) 50% sulphuric acid. (2) 15% sodium tungstate. (3) Freshly distilled ether, free from peroxide. (4) 2:4-dinitrophenylhydrazine, 1% dissolved in 10% H_2SO_4. (5) $KMnO_4$, solid and 2% aqueous solution. (6) Reagents for the determination of succinic acid (see Krebs [1937]).

Special apparatus required:

(1) Continuous extractors (Kutscher-Steudel, preferably with interchangeable joints). (2) Warburg-Barcroft manometers with conical flasks provided with centre chamber and sidearm. The sidearm should hold at least 1 ml. liquid. (3) Separating funnels, 50 ml. and larger sizes.

Removal of frothing substances. The ethereal extraction of aqueous tissue extracts or of urine is frequently interfered with by the presence of frothing substances which can be removed by treatment with tungstic acid. To 20 ml. aqueous solution (urine, tissue extract, dilute blood or serum) are added 1 ml. 15% tungstate and, drop by drop, with stirring, 1 ml. 50% H_2SO_4. The precipitate is filtered off after at least 30 min. and an aliquot of the filtrate is used for the determination. Precipitates which may appear in the filtrate on standing do not interfere with the further procedure.

Formation of the 2:4-dinitrophenylhydrazone. The amount of 2:4-dinitrophenylhydrazine which has to be added depends on the quantity of ketonic and aldehydic compounds present in the solution. It will usually suffice to add 1 ml. 1% solution to 5 ml. filtrate. A yellow colour of the aqueous phase after the first ethereal extraction indicates an excess of 2:4-dinitrophenylhydrazine.

The solution is allowed to stand for 30 min. before the extraction. The formation of the 2:4-dinitrophenylhydrazone is then complete.

Extraction. I have investigated the partition of α-ketoglutaric 2:4-dinitrophenylhydrazone between ether and 5% H_2SO_4 and find that the hydrazone is completely extracted if the solution is treated twice in a separating funnel with 1/5 vol. ether.

Treatment of the ethereal extract. The ethereal solution is evaporated on a steam bath and 2–5 ml. 2*N* NaOH are added to dissolve the residue containing hydrazones and hydrazine. The solution is quantitatively washed into a graduated measuring cylinder. The solution (about 20 ml.) is acidified with 50% H_2SO_4 so as to bring the acidity to about *N*. 3 ml. 2% $KMnO_4$ are then added. The solution is allowed to stand for 15 min. and, if the purple colour of the permanganate disappears, fresh oxidizing agent, either solid or in solution, must be added. The volume of the liquid is then read and the MnO_2 formed is filtered off. Succinic acid is extracted from an aliquot of the filtrate in the continuous extractor and determined as described before [Krebs, 1937].

Specificity. Substances which may interfere with the determination of α-ketoglutaric acid are succinic acid and such substances as may yield succinic acid on treatment with permanganate. To the latter group belong glutamic acid, α-hydroxyglutaric acid, arginine, ornithine, butyric acid, valeric acid, *n*-hexanoic acid, suberic acid. Table II shows the yields of succinic acid obtained from these substances under various conditions.

Table II. *Formation of succinic acid on oxidation of various substances by $KMnO_4$ in acid solution*

Room temperature, 5% H_2SO_4, 0·5% $KMnO_4$. The following substances yielded no succinic acid: acetic acid, citric acid, *iso*citric acid, *cis*- and *trans*-aconitic acids, *n*-octanoic acid, stearic acid, sebacic acid, proline, tyrosine, pyruvic 2:4-dinitrophenylhydrazone.

Substance	mg. used	Duration of treatment with $KMnO_4$	Amount of succinic found (μl. O_2 measured)	Yield %
Glutamic acid	17·3	15 min.	13	1
Glutamic acid	17·3	15 hr.	26	2
α-Hydroxyglutaric acid	7·4	15 min.	494	88
α-Hydroxyglutaric acid	7·4	15 hr.	515	92
Arginine monohydrochloride	12·1	15 min.	28	4
Arginine monohydrochloride	12·1	15 hr.	242	27
Ornithine monohydrochloride	81	15 min.	7·2	0·1
Ornithine monohydrochloride	81	15 hr.	188	3·3
n-Butyric acid	80	15 min.	24·5	0·5
n-Butyric acid	80	15 hr.	125	2·6
n-Valeric acid	100	15 hr.	16·3	0·15
n-Hexanoic acid	100	15 hr.	77	0·8
Suberic acid	87	15 hr.	37·5	0·3

A simple control experiment, however, is sufficient to determine the amount of succinic acid which is not derived from α-ketoglutaric acid. An aliquot of the solution in which α-ketoglutaric acid is to be determined is extracted with ether in the absence of 2·4-dinitrophenylhydrazine and the extract is otherwise treated in the same way as it is in the determination of α-ketoglutaric acid. This ethereal extract contains all the interfering substances, but only traces of α-ketoglutaric acid. If the succinic acid found in this control is deducted, the method determines only those ketonic or aldehydic compounds which yield succinic acid on oxidation with permanganate. α-Ketoglutaric acid is the only compound of this type known to occur in biological material, and the method is thus highly specific.

Only negligible amounts of succinic acid were found in the control determination when normal urine or tissue extracts were analysed so that the control is often unnecessary. It is not essential if even considerable quantities of glutamic acid, arginine or ornithine are present, since these substances are practically

insoluble in ether. A control must be carried out, however, if significant quantities of succinic acid or large amounts of lower fatty acids or of α-hydroxyglutaric acid are present.

Recovery experiments. Known quantities of α-ketoglutaric acid added to urine were recovered satisfactorily as shown in Table III.

Table III. *Recovery of α-ketoglutaric acid from human urine*

α-Ketoglutaric acid found in 100 ml. urine mg.	α-Ketoglutaric acid added to 100 ml. urine mg.	α-Ketoglutaric acid recovery	
		Found mg.	Calculated mg.
2·59	0·93	3·51	3·52
3·73	1·86	5·30	5·59
1·49	1·86	3·51	3·35

α-Ketoglutaric acid in human urine

All urines that were tested contained α-ketoglutaric acid, the quantities excreted varying between 10 and 40 mg. per day (Table IV). 100 ml. urine thus contain enough α-ketoglutaric acid for accurate determination.

Table IV. *α-Ketoglutaric acid in human urine*

Initials	Sex	Age	Clinical diagnosis	Quantity of urine 24 hr. ml.	α-Keto-glutaric acid excreted in 24 hr. mg.
C. W.	♂	24	Normal	625	15·5
J. W.	♂	44	Chronic nephritis	965	21·4
W. C.	♂	18	Normal	484	14·5
A. G.	♀	53	Diabetes mellitus, controlled with insulin	1120	10·2
E. S.	♀	50	Acute heart failure	1450	37·5
E. J.	♀	39	Diabetes mellitus, controlled with insulin	1600	39·2
M. S.	♀	54	Cardiac failure	480	12·0
I. S.	♂	59	Normal	690	25·7
J. H.	♂	17	Diabetes mellitus, untreated	790	11·8

α-Ketoglutaric acid in human blood serum

Two specimens of blood were examined. The serum (100 ml.) was deproteinized according to the Folin-Wu method (at *p*H 4·5) and the determination was made on the filtrate. The first specimen, from a case of polycythemia vera, contained 1·05 mg. α-ketoglutaric acid in 100 ml., the second, from a case of cardiac decompensation, 0·75 mg. in 100 ml.

Shortened procedure

If α-ketoglutaric acid is present in a solution free from succinic acid or those substances listed in Table II, the procedure may be shortened. The oxidation to succinic acid can be carried out in this case directly by addition of $KMnO_4$ after acidification of the solution. The free acid like its 2:4-dinitrophenylhydrazone yields succinic acid quantitatively. For instance 6·09 mg. α-ketoglutaric acid gave 930 μl. CO_2 and 4·86 mg. succinic acid (calc. 930 μl. CO_2, 4·91 mg. succinic acid). The oxidation is complete within 20 min. (20°, 5% H_2SO_4, excess of $KMnO_4$).

Succinic and α-ketoglutaric acids, if present together in moderate quantities, may be determined in the following way. An aliquot is directly extracted with ether and succinic acid is determined as described previously. Another aliquot is treated with $KMnO_4$; the additional succinic acid found in this fraction is equivalent to the α-ketoglutaric acid present, provided that interfering substances (see Table II) are absent.

SUMMARY

A method for the quantitative determination of α-ketoglutaric acid in aqueous solutions is described. α-Ketoglutaric acid is first converted into the 2:4-dinitrophenylhydrazone which is then extracted with ether and oxidized quantitatively to succinic acid with permanganate; the succinic acid is determined manometrically by means of succinic dehydrogenase. The method is applicable to quantities from 0·2 mg. upwards. Ten human urines contained between 10 and 40 mg. α-ketoglutaric acid per 24 hr. specimen.

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XX. THE FORMATION OF CITRIC AND α-KETOGLUTARIC ACIDS IN THE MAMMALIAN BODY

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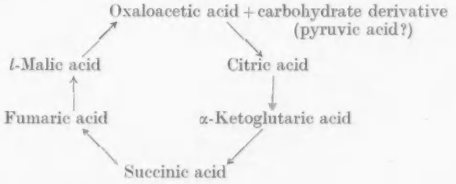
(*Received 1 December 1937*)

From experiments reported in a previous paper [Krebs & Johnson, 1937] we concluded that carbohydrate is oxidized in animal tissues through the following series of reactions:



The evidence in support of this scheme, the "citric acid cycle", was derived from work on isolated tissues. In the present paper experiments will be reported which supplement the previous evidence by experiments on the intact organism. It will be shown that considerable amounts of citric and α-ketoglutaric acids appear in the urine of rabbits and rats after intravenous injection of succinic, fumaric, *l*(−)-malic or oxaloacetic acids. Furthermore α-ketoglutaric and succinic acids are excreted if citric acid is injected. It is thus possible to demonstrate the occurrence of the "citric acid cycle" in the living organism.

Experiments of this kind were suggested to us by a paper of Orten & Smith [1937] who injected 21 different substances intravenously into dogs and studied the excretion of citric acid. They found that only succinic, fumaric, *l*(−)-malic and malonic acids increased the citric acid excretion. Our own experiments confirm and extend the findings of Orten & Smith and the results are in good agreement with the work on isolated tissues. The effects of succinic, fumaric, malic and oxaloacetic acids on the one hand and the effect of malonic acid on the other are due to two different mechanisms. The first group forms citric and α-ketoglutaric acids through the "citric acid cycle", whereas malonic acid acts as an enzyme poison.

The yields of citric and α-ketoglutaric acids are necessarily not quantitative and the evidence is therefore only of a qualitative nature. The question to what extent oxidations are brought about through the "citric acid cycle" can as yet not be decided by work on the intact normal organism, but only by experiments on isolated tissues.

Experimental procedure

Rabbits weighing about 2 kg. were used for most experiments. The solution was infused into an ear vein with the "slow perfusion apparatus" of Burn & Dale [1925]. The animal was kept in a metabolism cage and the urine was collected

in flasks containing a few ml. 20% H_2SO_4 as antiseptic. As a rule, 100 ml. 0·2 *M* solutions of the substrate were infused. Two-thirds of the acid equivalents were neutralized with NaOH, unless otherwise stated. The acid solutions prevent development of the alkalosis arising from the oxidation of the infused sodium salts of combustible acids. The rate of infusion was 1–2 ml. per min. No serious ill-effects were observed during or after the infusion except in the case of citric acid. Citrate and citric acid, as is well known, are highly toxic if injected intravenously [Salant & Wise, 1916]. We observed dyspnoea and convulsions after injection of only 10 ml. 0·2 *M* sodium citrate. The toxic effect was abolished by calcium and we have therefore used a mixture of disodium citrate and acid calcium citrate for the infusion.

A few experiments were performed on rats to which the substances were administered subcutaneously.

All determinations were carried out on 24 hr. specimens of urine. Citric acid was determined according to Pucher *et al.* [1936]. α-Ketoglutaric acid was estimated by the method of Krebs [1938]. Special care was taken to destroy interfering substances such as oxaloacetic acid before the determination of citric acid [see Krebs & Johnson, 1937]. Succinic acid was determined in the following way: the urine was deproteinized with tungstic acid and α-ketoglutaric acid was removed from the solution as dinitrophenylhydrazone, as described in the preceding paper. The aqueous phase was then freed from ether and treated exhaustively with permanganate. The volume of the solution was measured, the MnO_2 formed was filtered off and succinic acid was extracted from an aliquot of the filtrate and determined manometrically [Krebs, 1937]. The "succinic" acid found thus includes the succinic acid formed by oxidation with permanganate. The total quantity of "succinic acid" found in normal urine is too small to allow one to distinguish between preformed succinic acid and succinic acid formed by oxidation.

Results

Each type of experiment was carried out two or three times with uniform results. Typical results are recorded in Tables I and II and Figs. 1 and 2.

It will be seen that citric and α-ketoglutaric acids appear in the urine in considerable quantities after the injections. Assuming that the acids arise from the injected C_4-dicarboxylic acids through the citric acid cycle we calculate the following yields:

		Citric and α-ketoglutaric acids found	
Acid injected	Micromols.	Micromols.	Yield %
Fumaric	20,000	694	3·5
Oxaloacetic	20,000	886	4·4
l(−)-Malic	20,000	484	2·4
Succinic	20,000	154	0·8

If citric acid is injected, only a small fraction appears unchanged in the urine [see Östberg, 1931], for instance 13% in the experiment recorded in Table I. Thus only a small fraction of citric acid present in the body escapes oxidation and is passed into the urine; it is therefore justifiable to assume that very much more citric and α-ketoglutaric acids are actually formed in the body after the injection than are found in our experiments.

Malonic acid is known to be excreted unchanged; it is not metabolized. The work on isolated enzyme systems shows that the acid is an enzyme poison [Quastel & Wooldridge, 1929]. Malonate inhibits in the first place succinic dehydrogenase and we find accordingly a large output of succinic acid after

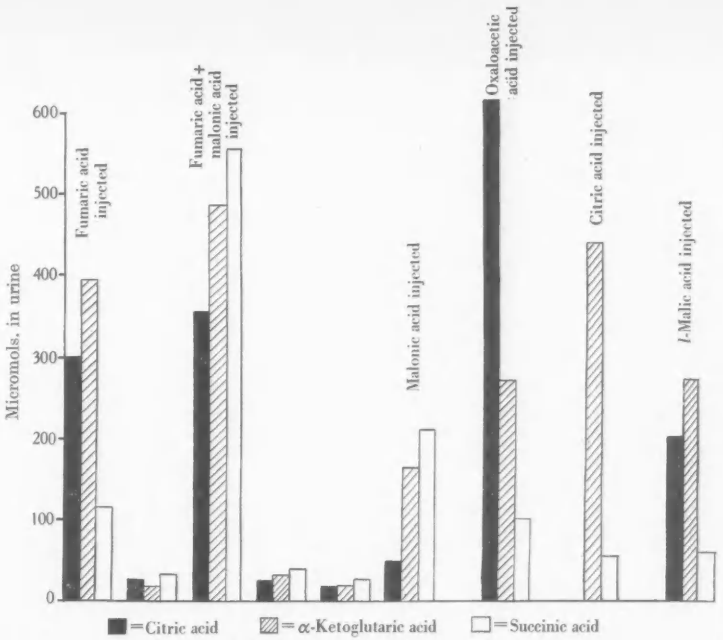


Fig. 1. Citric, α-ketoglutaric and succinic acids in the urine of rabbits after intravenous injection of fumaric, malonic, oxaloacetic, citric and *l*(−)-malic acids.

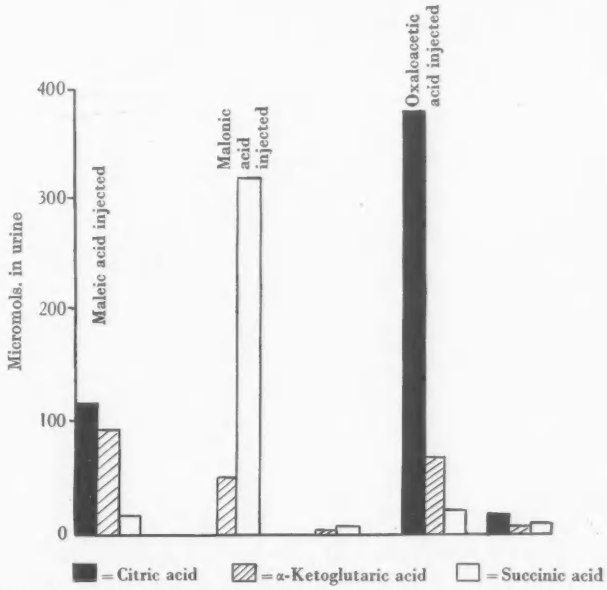


Fig. 2. Citric, α-ketoglutaric and succinic acids in the urine of rats after subcutaneous injection of maleic, malonic and oxaloacetic acids.

Table I. *Experiments on rabbits*

Wt. 1900–2000 g.; the acids injected were partly neutralized (see text) except malonic acid which was injected as neutral Na salt; "citric acid" was a solution of 3·8 g. anhydrous citric acid, 1 g. $CaCO_3$, 0·8 g. NaOH in 100 ml.

Rabbit no.	Day	Solution injected	Excreted (micromols.) in 24 hr. Citric acid	α-Keto-glutaric acid	Succinic acid
I	1	96 ml. *M*/5 fumaric acid	300	394	116
	2	Nil	26	18	32
	3	100 ml. *M*/5 fumaric acid *M*/10 malonate	355	487	554
	4	Nil	25	31	40
	5	Nil	17	18	25
	6	100 ml. *M*/5 neutral malonate	49	161	210
	11	100 ml. oxaloacetic acid	615	271	99
	12	Nil	208	72·2	59
II	1	100 ml. citric acid	(2310)	440	52
	2	Nil	25·5	12	2
	5	100 ml. *M*/5 succinic acid	84	70	(6300)
III	1	100 ml. *M*/5 *l*(−)-malic acid	201	273	59
	2	Nil	77	25	39

Table II. *Experiments on rats*

Four rats used for each experiment. Wt. in Exp. I: 218, 260, 225 and 207 g.; in Exp. II: 178, 184, 221 and 228 g.

Exp. no.	Day	Solution injected (per rat)	Micromols. excreted in 24 hr. Citric acid	α-Keto-glutaric acid	Succinic acid
I	1	Nil	—	1·3	5·4
	2	2 ml. *M* Na-malonate	—	51·4	324
	3	Nil	—	2·2	8·8
	4	Nil	9·7	5·3	12·4
	5	2 ml. *M* Na oxaloacetate	382	60·0	30·0
	6	Nil	17	4·9	8·0
II	1	0·5 *M* Na maleate	120	96·5	20·5
	2	Nil	—	12·8	8·7

injection of malonate. It increases too, to some extent, the excretion of citric and α-ketoglutaric acids. The magnitude of this side effect seems to vary in different species.

Greville [1936] and Weil-Malherbe [1937] have recently suggested that succinic dehydrogenase may be protected from malonate in the structurally intact tissue. Our experiments show, however, an inhibition of succinic dehydrogenase in the intact body.

Maleic acid causes an increased citric acid excretion in Orten & Smith's and our experiments (Table II). It is probable that maleic acid, too, is an enzyme poison, but we cannot exclude the possibility that it acts as a precursor like fumaric acid. Thunberg [1920] and Laki [1935] reported that maleic acid is metabolized in animal tissues.

Summary

1. Citric and α-ketoglutaric acids appear in the urine of rabbits and rats after injection of succinic, fumaric, *l*(−)-malic or oxaloacetic acids. This is considered to be evidence in support of the "citric acid cycle".

2. Malonate, injected into rabbits or rats, causes an excretion of succinic acid and, to a lesser extent, of citric and α-ketoglutaric acids. Since malonate is not metabolized in animal tissues its effect must be due to the inhibition of the enzymes concerned with the breakdown of succinic, citric and α-ketoglutaric acids.

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Note added 4 January, 1938. Breusch has recently questioned the existence of the "citric acid cycle" in muscle tissue [*Hoppe-Seyl. Z.* **250**, 262]. We are, however, unable to accept his arguments and shall discuss them in full in a subsequent paper.

XXI. THE DISTRIBUTION OF VITAMIN A AND FACTOR A_2. I

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The present paper is the full account of work briefly reported elsewhere [Lovern *et al.* 1937; Edisbury *et al.* 1937]. The main experimental result is that fish viscera, and notably the pyloric caeca and intestines, are often very rich in vitamin A. Large deposits of vitamin in the absorptive parts of the alimentary tract make it necessary to study the possibility that vitamin A plays an essential part in food assimilation in some species. This idea is discussed in a preliminary fashion, whilst other aspects of the subject such as the physical properties of visceral oils, vitamin D content, phospholipid content, glyceride composition etc., await further study.

Euler *et al.* [1928; 1929] showed that carotene displays vitamin A activity, in accordance with an earlier suggestion made by Steenbock. The accepted chemical structures and the known physical properties of β-carotene and vitamin A are consistent with a process

$$\underset{\beta\text{-carotene}}{C_{40}H_{56}} \xrightarrow{2H_2O} \underset{2\text{ vitamin A}}{2C_{20}H_{29}OH}$$

first recognized *in vivo* by Moore [1929, 1, 2, 3]. His proof that the administration of a diet rich in carotene results in the rat in a greatly increased accumulation of vitamin A in the liver has been confirmed and extended to fowls and rabbits [Drummond *et al.* 1930; Capper, 1930, 1, 2; Capper *et al.* 1931; Wolff *et al.* 1930].

Biological assays [Morgan *et al.* 1935; Pritchard *et al.* 1937; *M.R.C. Sp. Rep.* No. 202, 1935] carried out on rats suffering from vitamin A deprivation, show that β-carotene and vitamin A are weight for weight about equally effective in restoring growth. This suggests a quantitative conversion of β-carotene into vitamin A when the smallest doses of carotene are given which will produce a satisfactory response.

The important work of Wald [1934; 1935, 1, 2, 3; 1936, 1, 2; 1937, 1, 2] on the vitamin A cycle in low intensity vision reveals a great deal concerning the intimate mechanism of one process in which the vitamin is utilized.

The detection and estimation of carotene and vitamin A in large or small quantities are now on a fairly satisfactory basis [cf. Morton, 1935; 1937] when direct absorption spectra data and/or spectroscopic evaluation of the antimony trichloride colour test are possible, and the application of strict spectroscopic criteria to the promising synthetic materials obtained by Heilbron and others [Davies *et al.* 1935; Heilbron *et al.* 1936; Batty *et al.* 1937; Kuhn & Morris, 1937], will be awaited with interest. Although gratifying progress has been made, the general situation is still unsatisfactory in many ways:

I. Inadequate supplies of vitamin A are demonstrably a factor in widespread malnutrition, so that the value of the vitamin in preventive medicine is manifest.

On the other hand, the conditions for its effective use as a therapeutic agent are less clearly established. Vitamin A is often administered when there is no reason to suspect depletion of the normal liver reserves, and recent advances in the direction of a sharp criterion of suboptimal resources [cf. Jeans & Zentmire, 1934] are very welcome. There is some evidence of immobilization of liver reserves [Mellanby, 1934] and of beneficial effects (e.g. in disseminated sclerosis) not clearly connected with the relief of frank deficiency.

II. The known precursors of vitamin A all possess the β-ionone ring and the characteristic side chain, but it would be premature to assume either that no new provitamins A remain to be discovered or that the structure must be in large measure preformed before the *in vivo* transformation can occur. Neither the mechanism nor the site of synthesis is established. The claims to have achieved the conversion of carotene into vitamin A *in vitro* under the action of a liver carotenase are not in our experience verifiable [cf. Rea & Drummond, 1932; Woolf & Moore, 1932].

The work of Drummond and his colleagues on mammals [Drummond & Macwalter, 1933; 1934; Drummond *et al.* 1934] seems to eliminate the whole of the alimentary tract from the possible sites of synthesis *in vivo*. When carotene was introduced into the circulation, it was rapidly taken up by the liver and disappeared in a few days. Experiments involving partial hepatectomy followed by introduction of carotene showed that operation-shock of itself brought about a large fall in the vitamin A reserves of the remaining liver lobes. Suggestive as this phenomenon is from the point of view of vitamin A therapy, it prevents an acutely planned experiment from achieving its immediate object of settling whether the liver is or is not the site of vitamin A synthesis. The prevailing idea that provitamins A (carotene, etc.) ingested orally are absorbed from the intestine and conveyed to the liver for (possibly enzymic) hydrolysis to vitamin A, is at present no more than an attractive hypothesis. The objections to it are perhaps most serious for the general case of fishes (*vide infra*) where the vitamin A concentration attains levels difficult to reconcile with the intake of dietary vitamin A or known provitamins.

III. Another difficulty lies in the disparities in vitamin A content which emerge when different animals and fishes are compared. These disparities are very large, e.g.

	Parts per million vitamin A
Human liver	20
Rabbit liver	70
Halibut liver	2000

Differences of this kind recur within genera and species, although mammals seem to possess lower reserves than many fishes.

It is possible that some species accumulate vast reserves of vitamin in the same way as an experimental rat which is receiving carotene in quantities unnaturally large for a herbivorous animal. Such an idea would perhaps mean that the enormous deposits of vitamin A in, let us say, halibut, are in a sense accidental and without function, harmless possibly, but as irrelevant to nutrition as an intestinal calculus. It should be noted, however, that the diet of the halibut (mixed species of fish) is not appreciably different from that of other fish which do not contain such large quantities of vitamin A.

Alternatively it is necessary to examine the notion that the vitamin can fulfil a variety of functions. The diet of the carnivorous fish consists mainly of fat and protein, and the occurrence of vitamin A in the absorptive sections of the alimentary tract is enough to warrant investigation of the role it plays in

assimilation of either fat or protein or both. Its distribution runs suggestively parallel with the distribution claimed for fat absorption, whilst there is much evidence that at other sites it promotes the proper functioning of epithelial cells "working in harmony with proteins of high biological value" [Mellanby, 1934].

The conception of a large physiological response to a small quantum of accessory food factor is part of the classical notion of a vitamin; there is however no reason to exclude other functions, especially in a fish like halibut which contains many grams of a substance hitherto mentally associated with a minute fraction of a milligramme capable of relieving a condition of outspoken deficiency. When, as is so often said, the animal is to be regarded as the final court of appeal in vitamin research, it must be remembered that the statement has so far implied the prevention or cure of avitaminosis through the response to minimal doses. Unless we are to adopt the hypothesis of fortuitous accumulation, there are obvious attractions in the idea of grosser processes requiring much larger amounts than those needed to prevent or counteract recognized deficiencies. It will be useful at this stage to draw a theoretical distinction between (*a*) "classical" conceptions of the functions of vitamins, and (*b*) newer conceptions of functions involving relatively large absolute quantities of vitamin.

Clinical tests with concentrates might well be reviewed in the light of such a distinction.

IV. The quantitative conversion of carotene (or other provitamin) into vitamin A may not occur without hitherto undetected intermediate stages, a possibility which complicates the problem of recognizing the site or sites of the processes.

V. More immediate from the present point of view are the difficulties concerning the antimony trichloride colour test. The blue solution obtained with vitamin A shows $\lambda_{max.}$ 620mμ., displaced to 605mμ. in weak oils. Since 1929 repeated observations of a maximum near 693mμ. have been recorded [Morton *et al.* 1931; Heilbron *et al.* 1931; 1932; Edisbury *et al.* 1932; Lederer & Rosanova, 1937; Edisbury *et al.* 1937; Heilbron *et al.* 1937] and sharp bands at 635–640 and 660mμ. have occasionally been seen. These chromogens may be akin to vitamin A. They have given rise to speculation as to their structure, and misgivings have been felt that accepted ideas about the physiological roles of carotenoids and polyene alcohols are much too simple. The work of Wald [1937, 1, 2] on porphyropsin anticipates our own work [Edisbury *et al.* 1937]; together they suggest that the 693mμ. chromogen may become recognized as a new vitamin. In the present paper this chromogen is referred to as factor A_2. There is no evidence that its function as a vitamin extends beyond fishes.

VI. Lastly, the importance of the liver as the main depot for vitamin A is now greatly reduced. Some rather neglected observations by Rosenheim & Webster [1927] on eels suggested as much, whilst later work [Edisbury *et al.* 1937] confirmed a wide dissemination of the vitamin over the eel body as a whole. Non-liver tissues of other species have also been found to contain substantial quantities of vitamin A, often exceeding the total liver reserves (*vide infra*) [cf. Shorland, 1937].

The following notes are intended to assist the perusal of the experimental sections.

For purposes of calculation the criteria:

$E^{1\%}_{1\,cm.}$		
620mμ.	5000	Colour test
583	2600	
325	1600	Ultraviolet absorption

[Carr & Jewell, 1933; Morton, 1935]

may be taken to represent the intensities of the absorption maxima characteristic of practically "pure" vitamin A. Thus an oil showing

$E^{1\%}_{1\,cm.}$	620 mμ.
583	26
325	16

contains 1% of vitamin calculated as free alcohol (or correspondingly more if calculated as ester). Some slight revision may be necessary if various claims to have prepared rather purer materials are substantiated. For the present the above figures afford a convenient basis for comparison.

Factor A_2 has not been isolated in a state approaching purity, so that strictly quantitative criteria are lacking. Approximate criteria can be obtained from data on sterol-free concentrates containing over 75% of vitamin A:

$E^{1\%}_{1\,cm.}$ 693 mμ.	$E^{1\%}_{1\,cm.}$ 620 mμ.	% vitamin A	% factor A_2 by difference
671	4325	86·5	> 13·5
594	4250	85	> 15
720	4150	83	> 17
945	3813	76·2	> 23·8
1050	3820	76·4	> 23·6
1000	4000	80	> 20

Although it is not safe to assume that factor A_2 is the sole contaminant of these very rich vitamin A concentrates, substantial amounts of other impurities are very unlikely. The figures indicate that for 100% factor A_2 $E^{1\%}_{1\,cm.}$ 693 mμ. will be of the order of 5000.

Provisionally, therefore, $E^{1\%}_{1\,cm.}$ 5000 in the colour test can be accepted for both vitamin A and factor A_2 although it is recognized that the results may ultimately have to be recalculated.

Saponification technique. The simplest and probably most accurate evaluation of the total vitamin A in tissues is obtained by direct saponification of the whole material, or of a representative sample, by means of alcoholic KOH.

To every 3 g. of tissue add 1 ml. 60% aqueous KOH and 10–20 ml. absolute alcohol, and heat on a water bath. Loss of solvent may be avoided by adding more alcohol, but this is rarely necessary. The amount of alkali represents considerable excess; this is necessary to ensure complete disintegration of tissue and saponification of fat in 1–2 hr. Preliminary extraction of tissue in order to determine the oil content is best performed as a separate experiment, especially if the vitamin content is low. With small amounts of material it is not usually worth while to determine oil, and vitamin estimations carried out on oils rather than on direct non-saponifiable extracts may err on the low side. With oils of low potency, preliminary preparation of "non-sap." is essential, and 0·5 ml. 60% KOH and 10 ml. alcohol are used for each g. of oil. Saponification at 100° is continued for 5–15 min. The hot solution of saponification products (whether from tissue or oil) is diluted with twice its volume of cold water and extracted twice with 3 times its volume of freshly redistilled (peroxide-free) ether. Further extraction is only occasionally necessary. The combined extracts are washed as follows: water, dilute alkali, water, dilute alkali, water 3–4 times [cf. S.P.A. technique, *Analyst*, 1933, **58**, 203; Morgan *et al.* 1935; *M.R.C. Sp. Rep.* No. 202, p. 52]. Aliquot portions are taken for the colour test and for the measurement of ultraviolet absorption. The ether is distilled off and the slightly wet residue is made up to known volume for ultraviolet absorption with absolute alcohol. For the colour test, moisture is first removed by twice adding a few drops of alcohol and "blowing" with nitrogen or carbon dioxide from a cylinder, while the flask is warmed on a water bath. The dry residue is then dissolved in a known volume of pure chloroform. Small quantities (0·2–0·4 ml.) are treated with the antimony trichloride reagent and the blue colour is studied spectroscopically.

Vitamin A and factor A_2 in eyes

The determinations were made on light-adapted eyes, and retinae were not examined separately, since the object of the work was to ascertain the absolute amount of vitamin present in whole eyes. It was hoped that the information

gained would help to distinguish organs or sites of storage, and sites of utilization, in a process involving true vitamins.

It is unnecessary (and in fact undesirable) to isolate the fat before determining vitamin A in eyes. The whole eyes are treated with alcoholic KOH until disintegration is complete (1–2 hr.). The lenses separate cleanly at an early stage and are only slowly attacked by alkali. The choroid is even more resistant. The non-saponifiable fraction is extracted in the usual way (*vide* p. 121) and although the amount is barely visible, satisfactory tests are possible.

The extracts obtained did not all give unequivocal evidence of the presence of vitamin A; thus a batch of brown trout (*Salmo trutta* v. *fario*) eyes and the eyes from one specimen of sea bream (*Pagellus centrodontus*) yielded pale yellow extracts giving a slate blue colour with the antimony trichloride reagent. The blue solutions showed a faint absorption maximum at 590–595mμ. which indicated the presence of a little carotenoid, but scarcely any vitamin A. All the other non-saponifiable extracts from eyes gave, however, positive tests for vitamin A, the concentrations (expressed in parts per million of whole eye tissues) remaining remarkably constant over a number of species.

The eyes of goldfish (*Carassius auratus*) proved exceptional and yielded 10–20 times the "normal" quantity of vitamin A. The concentrations observed were in fact appreciably higher than in some liver tissues (e.g. a human liver). One specimen contained nearly as much vitamin A in the eyes as in the rest of its body. The rich extracts from goldfish eyes exhibit a well-defined ultraviolet absorption spectrum. This is naturally affected by extraneous absorption due to substances other than the main absorbing entity. The colour test also exhibits a well-defined absorption spectrum influenced in some degree by substances other than the principal chromogens, and there is evidence that one chromogen can interfere with another and cause partial inhibition. Extraneous absorption in the ultraviolet raises the intensity of absorption and inhibition in the colour test depresses the readings. In consequence of these two tendencies, a rather wide margin of uncertainty sometimes appears in the estimates of vitamin content (see Table I).

Goldfish eyes differed materially from those of sea fish in that the maximum in the antimony trichloride colour test occurred at 690–695mμ. rather than 610–620mμ. (green colour instead of blue). The non-saponifiable extracts from goldfish eyes, dissolved in absolute alcohol, exhibited ultraviolet absorption spectra with maxima at *ca.* 350 and 288mμ. as distinct from the single maximum at 325mμ. associated with vitamin A. It is well known [Edisbury *et al.* 1932; Pritchard *et al.* 1937] that under the action of alcoholic HCl the vitamin A molecule undergoes cyclization whereby the broad unresolved band at 325mμ. is replaced by a group of narrow bands:

$$\lambda_{\text{max.}}\ 392,\ 369,\ 350 \text{ and } 333\text{m}\mu.$$

Cyclization of the extract from goldfish eyes gave

$$\lambda_{\text{max.}}\ 391,\ 369,\ 349 \text{ and } 334\text{m}\mu.$$

The apparent identity of the cyclization products makes it clear that the structural difference between the 620 and the 693mμ. parent chromogens can only be small.

Wald [1937, 1, 2] has shown that the 693mμ. chromogen can replace vitamin A in the visual purple cycle. He refers to the pigment in the eyes of freshwater fishes as porphyropsin to distinguish it from rhodopsin. The 693mμ. chromogen also occurs in the livers and viscera of a great variety of sea fish, but is accom-

panied by a considerably greater quantity of vitamin A. In a number of freshwater fishes the proportions are almost reversed, particularly in the liver [cf. Lederer & Rosanova, 1937; Heilbron *et al.* 1937].

Vitamin A is sometimes replaced by the 693 mμ. chromogen, referred to as factor A_2, in fishes' eyes, liver and viscera (pyloric caeca, intestines, spleen etc.). The chromogen does not seem to occur in mammals and feeding tests on mammals will be difficult to interpret. A negative response to avitaminosis-A would be expected unless conversion into vitamin A occurred *in vivo*. In view of the structural similarity deduced for vitamin A and factor A_2 such a change is not impossible. Biological experiments on freshwater fishes are needed to ascertain the true role of factor A_2 apart from its function in vision.

Table I. *Vitamin A content of eyes*

	Number of eyes	mg. vitamin A per eye	Vitamin A concentration: parts per million of whole eye
Rabbit (*Lepus cuniculus*)	6	0·008	3
Haddock (*Gadus aeglefinus*)	6	0·003–0·0033	3·9–4·3
Whiting (*G. merlangus*)	10	0·01–0·014	4·5–6·1
Hake (*Merluccius vulgaris*)	12	0·022–0·023	2·6–2·7
Halibut (*Hippoglossus hippoglossus*)	4	0·0037	5·5
Herring (*Clupea harengus*)	36 (small)	0·0011	1·5
,,	21 (adult)	0·004–0·005	3·3–4
,,	12* (adult)	*ca.* 0·008	*ca.* 7
Goldfish (*Carassius auratus*), large variety	2	0·001–0·002	5–10
Goldfish (*C. auratus*), small variety	2	*ca.* 0·002	*ca.* 60
,, ,,	6	0·0016	50
,, ,,	10	0·0012	*ca.* 40
,, ,,	8	0·0019–0·0053	36–100
,, ,,	24	0·003	90

* Oil content 6·4%.

Distribution of vitamin A in the rabbit (Lepus cuniculus)

In this study it was hoped to ascertain the distribution of vitamin A in a mammal living on a natural diet. A herbivorous mammal was preferred to a carnivore since the latter would receive dietary vitamin A, whilst the rabbit receives only provitamin A (carotene) and that in relative abundance.

Three freshly shot rabbits were brought to the laboratory without delay and dissected. The different parts were combined and weighed (3 livers, 3 spleens etc.) and at once covered with alcoholic KOH. The non-saponifiable extracts were obtained and tested for vitamin A.

From experiments on rats, it is clear that when the need for vitamin A is acute, the mammalian organism achieves an almost quantitative conversion of β-carotene into vitamin A. On a carotene-rich diet the rat synthesizes and retains relatively large quantities of vitamin, whereas rabbits shot at midsummer and as replete with carotene as they would ever be in a state of nature, retain relatively little. This is in striking contrast with the data on halibut and other fishes.

The requirements of the rabbit in respect of vitamin A are very modest. It will be seen from Table II that about 95% of the total vitamin is present in the liver. In the female, the mammary gland is the next most important depot, or rather entrepot, since the store covers no more than a day or two's

Table II. *Vitamin A in the rabbit*

Tissues*	Wt. g.	$SbCl_3$ test	Vitamin A mg.	Concentration in tissue parts per million
Eyes	14·4	Clear max. 614 mμ.	0·046	*ca.* 3
Stomachs	58	Faint max. 585 mμ.	0·002 ?	—
Spleens	3·3	Negative	None	—
Hearts and lungs	53·3	Faint blue	0·005 ?	—
Mammary gland	72	Clear 614 mμ. max.	0·094	*ca.* 1
Pancreas	6·5	Faint max. 602 mμ.	0·0045	—
Mesentery	12·7	585 mμ. max.	0·01 ?	—
Gonads, Uteri, Bladders	13·3	Faint slaty blue	Trace only	—
Small intestines	150	Maxima at 617 and 585 mμ. clear	0·01–0·02	*ca.* 0·1
Large intestines	140	Maxima at 617 and 585 mμ. clear	0·3	2
Kidneys and suprarenals	34	Clear max. 615 mμ.	0·1	3
Livers	160	Good 617 mμ. band	11·0	*ca.* 70

* From 3 rabbits, except that only one mammary gland was used.

Liver vitamin A/Total vitamin A = 95%. 693 mμ. chromogen not observed.

supply for lactation. It may be significant that the large intestine contains approximately 2 p.p.m., suggesting a "classical" function akin to that obtaining in the eyes, which have 3 p.p.m.

Distribution of vitamin A in the herring (Clupea harengus)

The main interest of this work lies in the occurrence of relatively large quantities of vitamin A in the alimentary tract. In the herring the pyloric caeca are numerous and form a substantial, fairly compact mass.

The pyloric caeca are caecal appendages or outgrowths from the intestine, situated close to the pyloric extremity of the stomach and the intestinal apertures of the bile and pancreatic ducts. Absent from some species of fish, they are subject to extraordinary variations in number, size and arrangement. Many functions have been ascribed to the pyloric caeca; fat absorption seems to be one of the most important.

These appendages contain vitamin A in quantities comparable with those found in the liver, and if the portion of the intestines comprising the caeca is boiled with water a pleasant smelling oil is easily obtained and is found to be similar to cod liver oil in vitamin A potency.

The herring, as is well known, varies enormously in oil content; the sex cycle, the season and the diet being concerned in varying degree.

The figures given below may not be representative of the main catches. They make it clear however that more tests are needed on fish caught at different seasons and in different waters. The annual herring landings from British vessels (*ca.* 8,000,000 cwt.) represent more than twice the weight of the next largest catch (cod).

So far as the tests have gone, the viscera usually contain more vitamin A than the liver, although this is not always the case (see Exp. 4). The visceral oils are easily obtained but the potency is relatively low on account of the abundance of oil itself.

The 693 mμ. chromogen, factor A_2, is clearly present in herring oils, but the intensity of the band in the colour test is only *ca.* 1/6 that of the 620 mμ. band of vitamin A. A feeble colour test $\lambda_{max.}$ 590 mμ. is shown by material obtained

from stomachs, but is due either to minute traces of vitamin A in the presence of much natural inhibitor [cf. Lovern *et al.* 1931] or, more probably, to traces of carotenoids.

Table III. *Distribution of vitamin A in herrings*

Exp.	Material		Wt. g.	Wt. vitamin A mg.	Concentration of vitamin A parts per million	Wt. oil g.
1	1 entire fish		124	*ca.* 0·4	3·0	13
2	6 herrings	(Mean)	256	—	—	—
	Gonads	(Mean)	44·3	Negligible	—	—
	Eyes	(Each)	1·15	Trace	—	—
	Viscera, including liver and gills	(Mean)	13·5	0·12	9·0	0·40
	Body		—	None	—	—
3	1 herring ♀		283	—	—	—
	Body		205	None	—	—
	Liver		2·45	0·037	15	—
	Gills		5·1	Trace	—	—
	Gonads		64·1	Not tested	—	—
	Rest of viscera		6·7	0·08	12	—
4	1 herring ♀		—	—	—	—
	Body		172	None	—	—
	Liver		1·7	0·5	293	—
	Gonads		43	—	—	—
	Viscera		6·7	0·32	48	—
	Gall bladder		0·1	0·003	30	—
5	6 herrings		980	—	—	—
	Total viscera		101	2·6	25·8	45·4
6	20 herrings		—	—	—	—
	Pyloric caeca		42	3·36	80	—
	Livers and gall bladders		40	2·2	55	—
	Oesophagi and stomachs		36	(590 mμ.) Trace	—	—
	Intestines		20	0·4	20	—
7	20 herrings		—	—	—	—
	Pyloric caeca		47	7·2	153	5·5

Table IV. *Oils from herrings*

Exp.	Material	$SbCl_3$ test	Approximate vitamin A poteney in I.U./g.
2	Visceral oil	Normal; carried out on "non-sap" $\lambda_{max.}$ 620 mμ.	300
5	Visceral oil	Normal; tests on non-sap. calc. on oil $E^{1\%}_{1\,cm.}$ 693 mμ. 0·04; 620 0·29; 583 0·16	150
7	Oil from pyloric caeca	Tests on oil show inhibition like cod liver oil; tests on oil $E^{1\%}_{1\,cm.}$ 693 mμ. 0·8; 612 4·2; 580 3·1	2150

The preliminary work was carried out on material purchased from Liverpool retailers, and the later work was done on materials kindly furnished by the Staff of the Marine Biological Station (University of Liverpool) Port Erin, Isle of Man.

Distribution of vitamin A in salmon (Salmo Salar)

A fresh salmon (River Dee, Wales) was eviscerated and the "pluck" divided into three portions for vitamin A assay. Similar tests were made on the viscera of frozen Canadian salmon.

Table V

	One fresh salmon			Three frozen salmon		
	Wt. g.	Vitamin A mg.	Concentration p.p.m.	Wt. g.	Vitamin A mg.	Concentration p.p.m.
Pyloric caeca	25·9	35	1353	718	200	278
Liver	59·4	10	168	224	100	446
Rest of viscera	38	8·7	229	Intestines stomach	30 Trace	—

	Fresh salmon			Frozen salmon		
$E^{1\%}_{1\,cm.}$ *	693 mμ.	620 mμ.	583 mμ.	693 mμ.	620 mμ.	583 mμ.
Pyloric caeca	0·9	6·8	3·5	0·135	1·4	0·72
Liver	0·93	0·93	—	1·76	2·17	1·84
Intestines	0·16	1·16	0·56	0·2	2·1	1·1
Stomach	—	—	—	0·0012	0·0082	—

* Calculated on the wet weights immediately after dissection.

In the salmon family the pyloric caeca are very numerous and large in relation to the rest of the alimentary tract. They contain more vitamin A than the liver and the rest of the viscera combined. The stomachs contain only traces of vitamin, whereas the intestines have moderately large amounts.

The 693 mμ. chromogen occurs both in the liver and the alimentary tract, but whereas the ratio $E^{1\%}_{1\,cm.}$ 693/$E^{1\%}_{1\,cm.}$ 620 mμ. is approximately 1 for the liver, it falls to approximately 0·1 in the other viscera. Salmon livers are thus a good source of factor A_2, but the viscera yield oils corresponding closely with the more familiar fish liver oils. The 693 mμ. chromogen tends to preponderate over vitamin A in the livers of freshwater fishes [cf. Lederer & Rosanova, 1937; Heilbron *et al.* 1937]. In this respect salmon resembles the true freshwater fishes. The distribution of factor A_2 is shown below:

	1 fresh salmon	3 frozen salmon
Pyloric caeca	5	20
Liver	10	70
Rest of viscera	1·2	3

If $E^{1\%}_{1\,cm.}$ 693 mμ. = 5000 for factor A_2 the above figures represent mg. (but see p. 121).

The results on frozen salmon can be stated in another way.

Livers (224 g.) ≃ 1·5 g. non-sap. (partially sterol free) pyloric caeca (718 g.) ≃ 12 g. oil separated, residue yielded 1·34 g. non-sap.

		Liver "non-sap."	Pyloric caeca "non-sap."
Colour test	$E^{1\%}_{1\,cm.}$ 693 mμ.	236	64·2
		615 325	660
		580 163	360
Ultraviolet absorption	$E^{1\%}_{1\,cm.}$ 353 mμ.	50	
		327 89	
		288 50	

i.e. liver "non-sap." contains about 6% vitamin A and <6% factor A_2, while pyloric caeca "non-sap." contains about 13% vitamin A and <1·3% factor A_2.

Vitamin A and factor A_2 in trout (Salmo trutta *v.* fario; S. irideus)

Six brown trout (*S. fario*) were eviscerated soon after being caught in an Irish river and the "pluck" was at once placed under alcohol. The livers were later separated from the rest of the viscera, and the combined portions were weighed, saponified completely and the non-saponifiable matter extracted.

	Livers 8·3 g.			Other viscera 53 g.	
$*E^{1\%}_{1\,cm.}$	696 mμ.	0·157	Colour test	693 mμ.	0·04
	620	0·10	,,	620	0·027
	351 mμ.	0·08	Ultraviolet absorption	346 mμ.	0·023
	288	0·084	,, ,,	288	0·025

* Calculated on the weight of tissue.

The 693 mμ. chromogen was more powerful than the 620 mμ. chromogen in both fractions, and the ultraviolet absorption spectra showed two bands with maxima at 346–351 mμ. and 288 mμ. instead of the normal vitamin A maximum at 325 mμ.

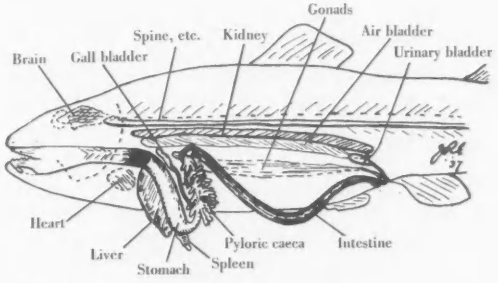

Fig. 1. A typical Teleostean (trout), showing pyloric caeca in relation to other organs.

A single brown trout was then dissected and various organs were worked up separately. The 693 mμ. chromogen again predominated, and it is estimated that the whole fish contained 0·25 mg. vitamin A and 0·5 mg. factor A_2.

	Liver		Pyloric caeca	Gonads
$E^{1\%}_{1\,cm.}$	690 mμ.	0·068	0·085	A small quantity only
	620	0·05	0·039	of vitamin A. 693 mμ.
	346	—	0·034	band not seen

The apparent correlation between the 346 mμ. maximum in direct absorption and the 693 mμ. chromogen is maintained.

A specimen of sea trout (*S. trutta*) was next examined.

	Liver		Pyloric caeca	Intestines
$E^{1\%}_{1\,cm.}$	690 mμ.	0·82	0·7	A trace only
	620	0·82	2·25	of vitamin A
	580	0·42	1·15	

The sea trout resembles the salmon rather than the river trout in respect of the amount and distribution of both chromogens.

A larger supply of material was obtained from the Liverpool Corporation Reservoir at Vyrnwy. By the courtesy of Captain Martin of the Lake Vyrnwy Hotel we were enabled to dissect the viscera of 8 rainbow trout (*S. irideus*) and 7 brown trout, freshly caught and placed in a refrigerator. The different portions of the viscera were immersed in alcohol and sent to the laboratory.

The pyloric caeca (68 g.) from 7 rainbow trout were treated with a small amount of alcoholic KOH at 100° for a period just long enough to disintegrate the tissues. On cooling, a pleasant smelling oil separated, part of which was decanted off and part extracted by means of redistilled ether. After removing solvent, the oil was centrifuged to remove tissue debris, and tested:

$E^{1\%}_{1\,cm.}$	693 mμ.	2·35
	620	9·4
	580	5·35

equivalent to 310 p.p.m. vitamin A in the wet tissue.

The oily product was thus several times richer in vitamin A (*ca.* 6000 I.U./g.) than cod liver oil, but the potency may have been raised by partial saponification of fat and accumulation of "non-sap" in the residual oil.

In order to ascertain how readily the 693 mμ. chromogen could be concentrated, the 15 livers were worked up as one batch:

161 g. liver ≃ 0·35 g. "non-sap." (sterol mixed with a red oil).

		"Non-sap."	
$E^{1\%}_{1\,cm.}$	690 mμ.	181	*ca.* 4·0 % factor A_2
	620	225	*ca.* 4·5 % vitamin A
	580	117	

The non-saponifiable matter from intestines (112 g.) gave

$*E^{1\%}_{1\,cm.}$	693 mμ.	0·12
	620	0·44
	580	0·23

* Calculated on tissue weight.

vitamin A preponderating markedly over factor A_2.

The stomachs (148 g.) gave on the same basis:

$E^{1\%}_{1\,cm.}$	693 mμ.	0·0016
	620	0·0045*

* i.e. just measurable quantities of both chromogens.

Table VI. *Distribution in Vyrnwy trout*

	Vitamin A		Factor A_2	
	mg.	p.p.m.	mg.*	$E^{1\%}_{1\,cm.}$ 693/620 mμ.
Stomachs (15)	> 0·15	1	*ca.* 0·05	1 : 3
Pyloric caeca (7 *S. irideus*)	21	310	5	1 : 4
Intestines (15)	10	90	2·5	1 : 4
Livers (15)	16	99	12	1 : 1·25

* See p. 121.

Making an allowance for the vitamin A contained in the 1 rainbow trout and the 7 smaller brown trout for which the figures were not obtained, it is clear that more than half the total occurs in the pyloric caeca and intestines.

In fact the liver contains only 1/3 of the total vitamin A, but 3/5 of the total factor A_2.

Although the concentrations in brown trout are lower than in rainbow trout (the Vyrnwy material was predominantly representative of the latter because the *S. fario* were much smaller than the *S. irideus*), it is still true that 2/3 of the vitamin A occurs outside the liver except in the sea trout.

Table VII. *Distribution of vitamin A in trout*

	One brown trout Vitamin A		Six brown trout from Ireland Vitamin A		Factor A_2	One sea trout Vitamin A		Factor A_2
	mg.	p.p.m.	mg.	p.p.m.	mg.*	mg.	p.p.m.	mg.*
Gonads	0·02	1·5	—	—	—	—	—	—
Pyloric caeca	0·15	17	—	—	—	1·8	138	0·6
Liver	0·7	10	0·166	20	0·4	4·1	164	4·1
Rest of viscera	None detected	0	0·27	5·2	0·26	—	—	—

* See p. 121.

The final experiment in this series on trout was on a rather larger scale, the object being to isolate a moderate quantity of visceral oil under mild chemical treatment.

A fishmonger was provided with a 2 l. brown glass bottle containing alcohol and was asked to fill it with the "pluck" from brown trout. The viscera (including livers) were left to stand for several days in the presence of a little alcoholic KOH. The material was then transferred to a large flask and brought to 100° for a few minutes. Although the tissue was only partially disintegrated, much oil came to the surface. It was extracted by means of redistilled ether, and a good yield of a pleasant smelling orange red oil resulted:

		Oil		
Colour test	$E^{1\%}_{1\,cm.}$	685 mμ.	0·72	
		636	0·74	
		598	0·63	
		557	0·50	
Direct absorption spectrum	$E^{1\%}_{1\,cm.}$	475 mμ.	0·12	
		448	0·15	Carotenoid?
		421	0·12	
		353	0·57	Marked inflexion
		325	0·6	,,
		282	1·0	Maximum

The oil contains it would seem, vitamin A, factor A_2 and a carotenoid.

The non-saponifiable extract gave

	Oil	
$E^{1\%}_{1\,cm.}$	691 mμ.	1·11
	620	0·61

whilst pigmented acids recovered from the soaps gave a green colour with antimony trichloride, $\lambda_{max.}$ 665, 590, 535 mμ., all transient.

A portion of the "non-sap." was cyclized by the use of alcoholic HCl and the recovered acid-free product was tested with antimony trichloride. Two bands were seen, one at 693 and one at 624 mμ., the latter being clearly defined and distinguishable from the 620 mμ. band of vitamin A. The 624 mμ. chromogen thus appears to be an artefact.

Earlier data on the sturgeon (*Acipenser sturio*) show that the bands at *ca.* 640, 595 and 560mμ. need not necessarily be accompanied by the 693mμ. band of factor A_2:

	Liver oil		Pancreatic oil		Peritoneal oil	
$E^{1\%}_{1\,cm.}$	617mμ.	39	640mμ.	0·24	640mμ.	0·12
	583	19	600	0·39	602	0·33
	325	18	565	0·35	568	0·3

Vitamin A in perch (Perca fluviatilis)

A preliminary study has been made of perch from Lake Windermere. All of the fish were fasting prior to death.

Table VIII. (*8 perch*)

	Total wt. g.	Chromogenic values calculated on tissue weights		
Livers	3·27	$E^{1\%}_{1\,cm.}$	693mμ.	0·04
			640	0·075
			595	Transient
			560	,,
Alimentary tracts	6·98	$E^{1\%}_{1\,cm.}$	693mμ.	0·008
			640	Transient green colour
Eyes	5·83	$E^{1\%}_{1\,cm.}$	610mμ.	> 0·003
		Qualitative test for vitamin A		
Other viscera (heart, spleen, gonads, mesentery)	20·3	$E^{1\%}_{1\,cm.}$	695mμ.	0·010
			636	0·011
			556	0·09

The colour test value for the eyes is unusually low (0·6 p.p.m.) and no factor A_2 was recorded. The fish had been fasting in a rather well lit aquarium and the results may not be entirely normal. The eyes are moreover unusually large in relation to the size of the fish. The bands at 693, 640, 595 and 560mμ., occurring even in the liver extract show a surprising chromogenic complexity, but the amounts of the chromogens must be very small indeed.

Table IX. *Distribution of vitamin A in turbot* (Psetta (Rhombus) maximus) (*12 fish*)

	Wt. g.	Wt. vitamin A mg.	Concentration vitamin A p.p.m.	% oil	Tests on oil		
Stomach*	450	3·01	6·7	0·79	$E^{1\%}_{1\,cm.}$	610mμ.	2·2‡
						572	2·2
Duodenum*	135	40·5	300	1·20	$E^{1\%}_{1\,cm.}$	693mμ.	<13§
						620	125
						583	66
Pyloric caeca†	62	>7‖	>115‖				
Ileum†	53·3	14·4	270				
Mesentery†	23·5	1·42	60				
Rectal caecum†	52·6	7·36	140				
Spleen†	30·9	0·18	6				

* Oils extracted at Aberdeen.

† Tissues placed in alcohol, saponified directly at Liverpool.

‡ The stomach oil is little better than cod liver oil and contains much colour test inhibitor.

§ The duodenal oil contains *ca.* 2·5% vitamin A and thus compares favourably with halibut liver oil. The whole of the intestines would probably give a similar oil.

‖ The turbot has two stumpy pyloric appendages; part of the material was used for other work, so that the above figures err on the low side.

Table X. *Distribution of vitamin A in conger eel* (Conger conger)

The tissues were extracted at Aberdeen, and part of the products sent to Liverpool for assay.

Tissue	Wt. g.	% oil	Wt. oil g.	$E^{1\%}_{1\,cm.}$ 620 mμ. on oil	Wt. vitamin A in tissue mg.	Concentration in tissue p.p.m.
Body plus head	8608	11·85	1020	0·154	32	3·72
Liver	116	14·2	16·5	24·0	79	681
Spleen	63	4·1	2·58	> 0·06	0·031	0·5
Stomach	65	0·8	0·52	0·86	0·088	1·35
Intestines	37	1·6	0·59	2·0	0·24	6·5
Mesentery	13	11·7	1·52	0·65	0·20	15·4
Gonads	425	59·2	251·6	0·58	29	68·2

Vitamin A in liver 79 mg.
Vitamin A elsewhere 61·6 mg.
Total 0·14 g.

The above results may be compared with earlier data [Edisbury *et al.* 1937] on the eels *Anguilla vulgaris* and *A. aucklandi*.

Table XI. *% total vitamin A occurring in different parts of the eel*

	Anguilla	*Conger*
Body (including head and skin)	35–75* <	23
Liver	20–60*	56
Viscera	2	0·4
Gonads	(Included in viscera above)	20

* Varies according to age and other factors.

In eels the gonads take the form of strips of adipose tissue, and from data on other fish it is probable that the greater part of the vitamin found in the "gonads" is present in the fat investing the tissue rather than in the gametes themselves.

Table XII. *Vitamin A in spotted ray* (Raia maculata). *River Dee Estuary, Cheshire*

Liver	0·7 p.p.m. vitamin A in tissue
Eyes	Mere trace vitamin A in tissue

The following organs all gave negative results for vitamin A: spleen, pancreas, gonads, stomach, spiral valve and intestines. The extraordinarily low vitamin A content of this large fish requires confirmation.

Table XIII. *Sunfish* (Orthagoriscus mola *or* mola mola)

One fish 3–4 cwt.; oils extracted at Aberdeen. Liver 17·4 kg.; 10·4 kg. oil containing *ca.* 0·75 g vitamin A, or 44 p.p.m. in the liver.

Negative tests for vitamin A were obtained on the following oils:

	% oil		% oil
Flesh	0·08	Stomach*	0·92
Cartilage	0·05	Duodenum*	0·47
Spleen	1·35	Ileum	0·49
Heart	0·95	Caecum	0·51
Mesentery*	0·97		

* The pyloric end of the stomach was filled with worms and much destruction had occurred.

Again, we have a large fish with very little vitamin A.

Table XIV. *Distribution of vitamin A in cod* (Gadus callarias) (*8 fish*). *Aberdeen*

Material	Wt. g.	Wt. oil g.	Vitamin A
Stomach	623	4·55	Nil
Pyloric caeca	331	4·70	26 mg.
Mesentery	28	0·37	Trace
Duodenum	137	1·03	Nil
Ileum	64	0·49	Nil
Caecum	59	0·48	Nil
Spleen*	—	1·3%	56 p.p.m. (tissue)

* Some missing.

	Pyloric caeca oil		Spleen oil
$E^{1\%}_{1\,cm.}$	693 mμ.	2·0	<5
	620	27·6	20
	583	15·6	12
	ca. 0·55% vitamin A		*ca.* 0·4% vitamin A

Visceral oils from cod may thus be obtained some 20 times richer in vitamin A than cod liver oil, but the present tests are essentially preliminary.

The distribution of vitamin A in the halibut (Hippoglossus hippoglossus)

Halibut liver oil is now produced on a large scale and is highly valued because it can be retailed with a potency some 60 times greater in respect of vitamin A than average medicinal cod liver oil. In order to maintain such a high potency it is necessary to blend rich oils with poorer oils since the vitamin content of halibut livers exhibits marked seasonal fluctuations [Lovern *et al.* 1933]. The richer oils are consequently in great demand.

It is shown in the present study that in addition to the liver, the alimentary tract of the halibut provides a notable and hitherto neglected source of vitamin A. The stomach is practically devoid of vitamin, but the pyloric caeca and intestines, though not rich in fat, contain very large quantities of vitamin A. If, therefore, the viscera are worked up by the ordinary methods of fat extraction, very potent oils (up to 33% vitamin A) can be obtained. The halibut possesses four pyloric appendages which are comparable with the intestine in size and weight. The spleen is relatively large and contains a substantial quantity of vitamin whilst the mesentery is a by no means negligible source (*vide* Table XV).

The first series of experiments concerns the distribution of vitamin A between the liver and the "viscera", i.e. stomach, spleen, intestines, mesentery etc., worked up together. The second series eliminates the stomach as a source and shows that most of the "non-liver" vitamin A occurs in the pyloric caeca and intestines. The third series carries the distribution into the different portions of the intestines. Work is well advanced on the distribution in the epithelial, areolar and muscle tissues in the intestine.

Series 1. Eight fish (A–H) were caught in Shetland waters in May 1937 by the crew of the research vessel (Torry Research Station). The three smallest fish (F, G, H) were treated as one and labelled I.

The "pluck" or total viscera from each fish was divided into two portions, liver and other viscera, and worked up separately for oil. 12 samples were thus obtained: liver oils A–E and I, "visceral" oils A–E and I.

Table XV shows (*a*) the variability of the livers in respect of both fat and vitamin A, (*b*) the variability of the fat and vitamin A content of the "viscera", (*c*) that the amount of vitamin in the "viscera" is of the same order as that in the liver and (*d*) the high potency of the "visceral" oils.

Table XV. *Distribution of vitamin A in halibut*

	Series 1					
Fish ...	A	B	C	D	E	I
Wt. fish kg.	45·4	45·4	28·6	23·6	26·9	36·3
liver g.	1030	540	400	340	510	910
liver oil g.	203	70	34	—	—	205
vitamin A in liver oil g.	3·84	1·53	2·1	—	—	0·22
% vitamin A in liver oil	2·0	2·2	6·3	4·6	0·33	0·11
Wt. viscera g.	1670	1470	760	850	1040	1060
oil from viscera g.	36	27	15	15	17	20
vitamin A in oil g.	2·88	1·51	1·05	0·9	0·48	0·32
% vitamin A in oil	8·0	5·6	7·0	6·0	2·8	1·6

	Series 2	
Fish ...	A′	B′
Wt. viscera g.	3050	2450
oil from viscera g.	45·5	31·2
vitamin A g.	4·13	4·003
% vitamin A in oil	9·1	12·62
Wt. stomach g.	2073	1784
oil from stomach g.	11·74	11·06
Vitamin A	Negligible	
Wt. mesentery g.	207	155
oil from mesentery g.	5·08	3·8
vitamin A mesentery g.	0·055	0·085
% vitamin A in oil	1·2	2·2
Wt. spleen g.	63	45
oil from spleen g.	1·52	1·02
vitamin A in oil g.	0·007	0·018
% vitamin A in oil	0·45	1·8
Wt. intestines g.	707	467
oil from intestines g.	27·15	15·32
vitamin A in oil g.	4·07	3·90
% vitamin A in oil	15	25·5

	Series 3	
Fish ...	C′	D′
Wt. intestines g.	688	573
oil from intestines g.	33·65	27·28
vitamin A in oil g.	9·22	6·87
% vitamin A in oil	27·4	25·2
Wt. pyloric caeca g.	369	321
oil from pyloric caeca g.	22·6	16·7
vitamin A in oil g.	5·94	4·01
% vitamin A in oil	26	24
Wt. duodenum g.	112	116
oil from duodenum g.	4·67	6·61
vitamin A in oil g.	1·35	1·72
% vitamin A in oil	29	26
Wt. ileum g.	171	86
oil from ileum g.	6·12	3·41
vitamin A in oil g.	1·9	1·06
% vitamin A in oil	31·5	31·5
Wt. caecum g.	36	50
oil from caecum g.	0·26	0·56
vitamin A in oil g.	0·031	0·084
% vitamin A in oil	12	15

Series 2. Two large halibut A′ and B′ were used for determining the distribution of vitamin A in the alimentary tract. The livers were not used. The averaged results showed:

	% oil in wet tissue	% vitamin A in oil
Stomachs	*ca.* 0·6	Negligibly small
Mesenteries	2·5	1·6
Spleens	2·3	1·0
Intestines including pyloric caeca	3·3	19·0

In these fish some 98% of the "visceral" vitamin A was therefore localized in the intestines. Stated in another way, by working up the intestines only, 80% of the weight of viscera was discarded at the cost of losing half the fat and 1/50 of the vitamin. By rejecting the stomach only, 1/3 of the weight of material was retained, with 2/3 of the fat and nearly all the vitamin.

The "visceral" oil obtained in this way may contain 25% of vitamin A ($C_{20}H_{29}OH$) or about 50% of vitamin A ester (if it exists wholly as ester).

Series 3. The distribution in the intestines of two large halibut C′ and D′ was now determined. The pyloric caeca were extracted separately and the intestines were divided into three portions, two corresponding approximately with a duodenum and an ileum, and a third comprising the rectal portion carrying a cloacal caecum.

Very high potencies were again observed, with a very interesting distribution of the vitamin:

	% total intestinal vitamin A	Relative wt. of tissues expressed as % total wt. intestines
Pyloric caeca	61	55
Duodenum	18·6	18
Ileum	19·7	20
Rectal (cloacal) caecum	0·7	7

A rough parallelism obtains between weights of vitamin and tissue for the pyloric caeca, duodenum and ileum, but there is an abrupt fall in vitamin A content at the rectal end of the intestine. It is suggestive that in the stomach, which is said to be concerned with disintegration and digestion rather than absorption of food, there is no vitamin A, and that the terminal portion of the intestine, which is mainly concerned with excretion, is relatively poor in vitamin, whilst the intermediate parts of the alimentary tract are very rich.

The oils from the ileum appeared to contain 33–37% of vitamin A when judged by the colour test and 31–32% on the basis of ultraviolet absorption. This is a real discrepancy which will be investigated further.

Table XVI. *Spectroscopic data on oils from halibut viscera*

	Intestines		Pyloric caeca		Duodenum		Ileum	
$E^{1\%}_{1\,cm.}$ 693 mμ.	38	55	68	60	74	90	84	66
620	750	1280	1313	1200	1450	1280	1600	1872
583	410	650	687	650	745	774	832	953
325	240	406	440	373	460	360	500	500

Ratio $E^{1\%}_{1\,cm.}$ 693 mμ./$E^{1\%}_{1\,cm.}$ 620 mμ.

Livers	*ca.* 1 : 6
"Viscera" (stomach, mesentery intestines)	1 : 10 (mean)
Intestines only	*ca.* 1 : 20
Pyloric caeca	*ca.* 1 : 20
Duodena, ilea, cloacal caeca	1 : 14–1 : 28 (variable)

Table XVII. *Distribution of vitamin A in halibut viscera*

(*a*) Total vitamin A g.

Fish ...	A′	B′		C′	D′
Mesentery	0·055	0·085	Pyloric caeca	5·95	4·01
Spleen	0·007	0·018	Duodenum	1·35	1·72
Stomach	Nil	Nil	Ileum	1·9	1·06
Intestines	4·07	3·90	Cloacal caecum	0·03	0·084
Total	4·132	4·003			
Intestines account for	98·5%	97·4%			

(*b*) Wt. vitamin A/wet wt. tissues, p.p.m.

Liver	*ca.* 2500 possibly 500–10,000 in extreme cases
Stomach	Very low
Mesentery	400
Spleen	230
Pyloric caeca	14,420
Duodenum	13,410
Ileum	11,500
Cloacal caecum	1,340

Preliminary tests showed that by the time the material was available for study the vitamin A of the intestines was not confined to the innermost epithelial layers.

Discussion

(1) A satisfying interpretation of all the foregoing data is not yet possible. An attempt will be made to state the various problems clearly; this involves perhaps uncritical acceptance of the views of workers whose experience concerns experimental fields different from our own, but this may be unavoidable.

Granted that most of the larger fish are carnivorous, the mechanism of food assimilation in fishes will be primarily concerned with the metabolism of protein and fat rather than carbohydrate. The comparative poverty of some species in respect of vitamin A in the alimentary tract suggests that the substance is not a *sine qua non* for assimilation of either protein or fat. The richness of other species in vitamin, and the sharply selective distribution over the different regions of the alimentary tract, suggests on the other hand participation of vitamin A (possibly as an intermediary) in food assimilation.

It is known that in the eye vitamin A functions in loose association with protein, whilst its association with fat is too well known to need emphasis. The acceptance of the idea of plurality of functions seems inevitable for vitamin A.

Fat absorption

The physiology of fat digestion and absorption in fishes has been studied very little considering the importance of fat in the diet of most species. Among the more outstanding papers reference may be made to the work of van Herwerden [1908], Greene [1912, 1, 2; 1913] and Dawes [1930].

Van Herwerden studied mainly the gastric processes. He demonstrated the presence of active lipolytic enzymes in gastric mucosa and showed that the fat droplets normally occurring in stomach epithelia were absent from fasting fish.

Greene carried out histological studies on the fat-absorbing function of the alimentary tract of the King salmon (*Oncorhynchus tschawytscha*). The amount of fat taken up by the mucous lining membrane of the stomach is "quite sufficient to form a very striking picture" but is "not anywhere near so great in amount as that shown by the mucosa of other portions of the alimentary tract". The pyloric division of the stomach is much more active in fat absorption than the cardiac division, and in the pyloric caeca the process takes place abundantly. "Whatever else these organs accomplish, it is perfectly clear that absorption of fat is one of their chief functions." Fat is also readily absorbed by the intestinal epithelium of salmon. Greene pictures the fat components as diffusing through the free walls of columnar epithelial cells prior to resynthesis, and he finds that the role of the stomach in fat absorption tends to decrease with increasing age of the fish.

Dawes, working on plaice (*Pleuronectes platessa*), arrived at very similar conclusions. Absorption of fat in the stomach could be observed for the first food taken after fasting. When however the fish were feeding freely, the stomach played a minor part in fat absorption. The pyloric caeca were definitely regarded as sites of fat synthesis and absorption, the histological work showing the tips of the mucosal folds to be specially effective. The duodenal and intestinal epithelia showed no signs of fat if the fish had been fasting, but the cells were loaded with fat droplets when food was abundant. Even the rectum was found to be capable of some fat absorption.

Sinclair [1929], in his work on fat absorption in the mammal, adduces evidence to the effect that "within the epithelial cells of the intestinal mucosa, there is

Table XVIII. *Distribution of vitamin A in relation to sites of fat absorption*

Stomach	Fat absorption can occur to a minor degree in the normal state	Vitamin A is either absent from the stomach or present in amounts which are small in both a relative and an absolute sense
Pyloric caeca	Very definitely sites of fat synthesis and absorption [Greene; Dawes]	In many species the pyloric caeca contain a substantial amount of vitamin A which may be large in an absolute sense and is apparently always large in relation to the total resources of the entire fish
Intestines	Also very effective in fat absorption, at any rate in some species	Frequently rich in vitamin A
Rectal portion (cloacal caecum etc.)	Much less effective	Vitamin A, when present is in much smaller concentration than in other parts of the intestines

a 'specific' phospholipid which occupies an intermediary position between fatty acids and neutral fat

$$\text{fatty acids} \rightleftharpoons \text{phospholipid} \rightleftharpoons \text{neutral fat}",$$

and approximate constancy in the amount of this lipid is essential to his hypothesis [cf. Verzàr & Laszt, 1934, 1, 2].

Dawes reviewed his own studies in relation to Sinclair's views. He obtained negative tests for phospholipid under circumstances such that positive results might have been expected if Sinclair's hypothesis were valid for plaice. A negative histochemical test for phospholipid is inconclusive [Carleton, 1926] and Dawes' experience may be given in his own words:

"It may be that the technique does not favour detection of the 'specific' phospholipid or that the groups of molecules are too small to be visible under the highest powers of the microscope, but in any case it is not possible to present evidence which supports this (i.e. Sinclair's) hypothesis."

If it is fair to conclude that the phospholipid mechanism is not demonstrable for plaice, it is equally fair to conclude that a mechanism involving vitamin A is not demonstrably applicable to mammals. The paucity of vitamin A in the alimentary tract of the rabbit is in harmony with the work of Green [1934, 1, 2] on rats, wherein he showed that although vitamin A deficiency brings about a large decrease in the esterase content of blood serum, fat metabolism is not obviously altered, since absorption, mobilization, desaturation and oxidation of fats continue in the absence of vitamin A.

The distribution of vitamin A in the alimentary tract of many fishes suggests a mechanism akin to Sinclair's, with the vitamin playing the part of intermediary. The main difficulty with such an idea is the enormous disparity in the intestines of different species. Alternative mechanisms for fat absorption seem inescapable, unless the parallelism between vitamin A distribution and fat assimilation is fortuitous.

(2) In their comprehensive report on cod liver oil, Drummond & Hilditch [1930] discuss the origin of vitamin A in the liver:

"...results were somewhat surprising in that they revealed that the commonest foods of the cod were much poorer in these substances (vitamins A and D) than had been suspected. To what, then, are the relatively large stores of vitamin A normally found in the liver of the cod to be attributed? There are two alternative explanations that come to the mind. In the first place,

there is the possibility that, although the amount of both vitamins in the daily food of the cod may be small, it may be retained in the liver so effectively that by the time the fish is mature the accumulation will be considerable. Alternatively there is the less attractive theory that the organism of the cod possesses the power to synthesize both vitamins A and D. For the time being, there is insufficient evidence to enable us to judge between these two theories, or to advance a more satisfactory one and further investigation is required to throw more light on the matter."

Later work [Drummond & Gunther, 1934] only served to increase the difficulties, for the zooplankton, an essential link in the hypothetical chain from the carotene of diatoms to the vitamin A of fishes, was repeatedly found to give negative or almost negative tests when fed to rats on a vitamin A-free diet, although diatoms gave positive results.

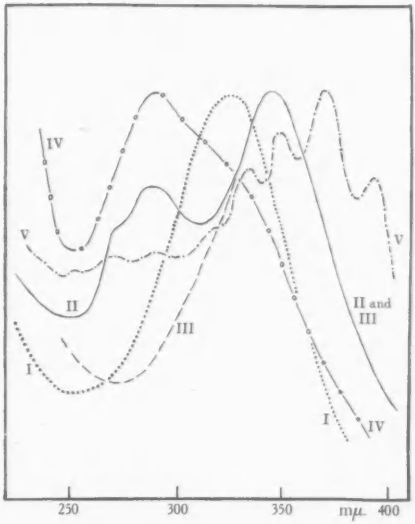

Fig. 2. I. Ultraviolet absorption spectrum of vitamin A; single maximum at 325 mμ. II. Typical ultraviolet spectrum of a material rich in factor A_2; the principal maximum is displaced progressively towards 350 mμ. as the ratio A_2/A increases. Note the subsidiary maximum at 285–290 mμ., and inflexion near 275 mμ. III. Fraction from II, soluble in 83% methyl alcohol. Selective absorption at 285–290 mμ. has been eliminated, without affecting the 350 mμ. band. IV. Fraction from II, insoluble in 83% methyl alcohol. The absorption is predominantly due to the 285–290 mμ. substance (A_3 ?). V. Cyclization product obtained by treatment of either I or II with $N/30$ alcoholic hydrogen chloride for 20 min. Narrow bands are seen with maxima at 392, 369, 350 and 333 mμ., and traces of ill-defined selective absorption further in the ultraviolet.

If the problem was regarded as acute for the cod, it is much more so for the halibut with its hundred-fold greater liver reserves [Lovern *et al.* 1933; Lovern & Sharp, 1933]. The discovery of large stores of vitamin A in the alimentary tracts of fishes complicates the position, for on the one hand it roughly doubles the recognized vitamin A intake in carnivorous fishes, and on the other hand it doubles the amount to be accounted for.

The contrast between for example a 4 cwt. sunfish and a large halibut is so striking as to render the claims of the "less attractive theory" more insistent.

(3) The vitamin A-like substances (i.e. polyene alcohols) present in the non-saponifiable extracts of oils, are evidently more numerous than is generally recognized:

Table XIX. *Polyene alcohols in non-saponifiable extracts of oils*

		Visible or ultraviolet maximum	$SbCl_3$ colour test maxima	Occurrence
Vitamin A, $C_{20}H_{29}OH$	Δ 5	325 mμ.	620 mμ. (605 mμ.) Blue colour	In eyes, liver, intestines, spleen, mesentery, etc. (*vide supra*)
Factor A_2	Δ 5	350 mμ. (288 mμ. ?)	693 mμ. Green colour	Do.
Chromogen(s)	Δ ?	?	635–640 mμ. 595 mμ. 557 mμ.	Sturgeon pancreatic and peritoneal fats, perch livers and viscera, trout viscera, mammalian blood?
Contaminant of mammalian "non-sap"	Δ 4 ?	286 mμ.	583 mμ.	Sheep liver, whale liver, some fishes
Chromogens	Δ ?	? Narrow bands 350–400 mμ. ?	660 mμ. 624 mμ.	Laboratory artefact(s)?
Acidic pigments (astacene type)	—	450–500 mμ.	Transient and feeble	Many zooplankton and some fishes

The above table is presented in order to assemble the known facts and to throw into relief the gaps in the evidence.

From the properties of the various chromogenic constituents, it is probable that the substances are polyene alcohols differing mainly in the number of conjugated double bonds. The fission *in vitro* of carotenoids at particular double bonds has eluded organic chemists; this is the more unfortunate since the properties of vitamin A analogues with one —CH=CH— more or less in the side chain might well clarify the whole problem of the natural chromogens.

The evidence now available suggests, but does not prove, that the 286 mμ. contaminant has one —CH=CH— group less than vitamin A and factor A_2 one more. The appreciably lower stability of factor A_2 to ultraviolet radiation is consistent with this hypothesis.

Summary

Whole eyes from a number of species of fish contain 3–7 p.p.m. vitamin A. Goldfish eyes are much richer and contain also the factor A_2 as shown by the 693 mμ. band in the colour test and the 345–350 mμ. maximum in the ultraviolet. Factor A_2 tends to replace vitamin A in freshwater fishes.

In the rabbit, the liver, with *ca.* 70 p.p.m., is the main vitamin A depot. Only traces were found in other organs and factor A_2 was not detected.

The herring contains relatively large quantities of vitamin A in the alimentary tract, particularly in the pyloric caeca. The latter may yield oil richer in vitamin A than cod liver oil.

Salmon pyloric caeca may contain more vitamin A than the livers of the same fishes. The non-saponifiable matter from the livers of frozen salmon contained 6% vitamin A and *ca.* 6% factor A_2, whereas a similar fraction from the pyloric caeca yielded 13% vitamin A and 1·3% factor A_2. The stomach contained only traces of vitamin A.

Trout also contain vitamin A and factor A_2 in the liver, pyloric caeca and intestines. Factor A_2 predominates over vitamin A in the brown trout and in the rainbow trout the ratio factor A_2/vitamin A is highest in the liver. The alimentary tract (especially the pyloric caeca) contains more vitamin than the liver.

Perch also contain vitamin A and its congeners at sites other than the liver.

Turbot contain little vitamin A (6–7 p.p.m.) in the stomach, but the remainder of the alimentary tract is relatively rich.

The conger eel stores about half its stock of vitamin A in the liver, the other half, apart from traces distributed widely, occurring in the body fat and the adipose tissue of the gonads.

Two large fish, one a spotted ray and the other a sunfish, contained surprisingly little vitamin A at any site.

In the cod, the pyloric caeca yield a few per cent of oil, the potency of which may be 10–20 times greater than that of the average cod liver oil.

The halibut has no vitamin A in the stomach but contains a very large quantity in the pyloric caeca and the intestines. Some intestinal oils contain 60–70% of vitamin A esters. The distribution of vitamin A in halibut intestines runs parallel with the weight of tissue except in the post-absorptive rectal region.

The possibility that vitamin A participates in the process of fat exchange or assimilation is considered in relation to published histological studies, and the significance of vitamin A in the alimentary tract is discussed in relation to its origin and its function; various difficulties are pointed out.

The position concerning vitamin A congeners is reviewed with particular reference to the 693mμ. chromogen (factor A_2). The available information suggests strongly that it is a polyene alcohol akin to, but more unsaturated than, vitamin A. The distribution of factor A_2 is consistent with the sharing of some at least of the functions of vitamin A in fishes generally and fish from fresh water more especially.

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XXII. THE ACTIVATION OF FEMALE SEX HORMONES

I. OESTRONE AND ITS ESTERS. THE OUTPUT OF ACTIVITY AND EFFICIENCY COEFFICIENT

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The increase of activity of sex hormones by artificial means is a problem of growing interest. Considerable progress has been made, particularly in the field of the male sex hormones, and a number of ways have been discovered which lead to the elucidation of the problem. It has been found that the method by which the hormones are administered, whether orally, subcutaneously, intravenously or percutaneously, plays an important part. With subcutaneous application the effect can be influenced in a number of ways, e.g. by the nature and quantity of the solvent used for the injection, by the addition of certain activators from the series of organic acids and alcohols, such as palmitic acid or stearyl alcohol and particularly by the transformation of the free hormone into suitable esters.

As extensive experiments in the series of the male sex hormones have shown [Miescher and collaborators, 1936, 1–3; 1937, 1, 2; Ruzicka & Wettstein, 1936; Deanesly & Parkes, 1936], such measures produce quite different results according to the substance used. The most favourable conditions must be ascertained for each case. The same applies also to the series of the female hormones.[1] In this paper the results of our experiments in the oestrone series will be reported. In a later publication oestradiol and its esters will be dealt with.

Table I

Oestrone esters	M.P. °C.	Threshold value S_r rat oestrone = 0·7 γ	Threshold value S_o based on oestrone* = 0·1 γ
Acetate	125–126	0·85	0·1
Propionate	134–135·5	0·85	0·1
n-Butyrate	101–102·5	1·25	0·2
*iso*Butyrate	120–121	1·5	0·2
n-Valerate	100–101	1·5	0·2
n-Hexanoate	94–94·5	3·5	0·5
n-Octanoate	70–71	4	0·6
n-Decanoate	71–71·5	6	0·9
Laurate	69·5–70	35	5
Palmitate	75·5–76	75	11
Stearate	81·5–82·5	75	11
Benzoate	220–222	3	0·4

* 0·1 γ oestrone = 1 I.U.

[1] Preliminary communication by Miescher *et al.* [1937, 3]. A report was made on the first results at the meeting of the Biochemical Society in London on 11 December 1936. The experimental results were submitted to the Swiss Medical and Biological Society in Geneva on 28 August 1937. See also Miescher [1937].

In addition to experiments with free oestrone we carried out extensive investigations with the esters shown in Table I.

Of these esters only the acetate and benzoate have been previously described by others. A report on the preparation of the remaining esters has been published elsewhere [Miescher & Scholz, 1937]. Oestrone *n*-octanoate and oestrone laurate are new.

Oestrone n-*octanoate.* A solution of 1 g. oestrone in 4 ml. pyridine was mixed with 1·5 mol. *n*-octanoyl chloride and the whole kept overnight. To finish off the reaction the solution was heated to 70° for 1 hr. After cooling it was acidified and extracted with ether. The ethereal solution was washed with dilute acid, dilute Na_2CO_3 and water; it was then dried and filtered and the solvent distilled off. The residue crystallized and was recrystallized from hexane and aqueous ethanol. The oestrone *n*-octanoate melts at 70–71°. (Found: C, 78·69; H, 9·25%. $C_{26}H_{36}O_3$ requires C, 78·73; H, 9·16%.)

Oestrone laurate. A solution of 400 mg. oestrone in 2 ml. pyridine was mixed with 1·5 mol. lauryl chloride and the reaction conducted as above. The working up was the same. The resulting oestrone laurate was recrystallized several times from methanol and ethanol. The M.P. is 69·5–70°. (Found: C, 79·48, 79·55; H, 9·75, 9·71%. $C_{30}H_{44}O_3$ requires C, 79·58; H, 9·80%.)

(I) *Test methods*

The effect of the compounds tested by us was determined quantitatively both in the oestrus test and on the uterus. As in our investigations regarding the activation of the male sex hormones we did not limit ourselves to the determination of the threshold values. The study of larger doses, particularly taking into consideration the time course of the effect, was found to be much more important.

Oestrus test. Castrated rats weighing about 120 g. were used. For the oestrus test we administered systematically the determined quantities of substance subcutaneously in two equal doses on consecutive days. Each dose of substance was dissolved in 0·5 ml. sesame oil. The day of onset of oestrus as well as its duration in days were determined. Assessment was made only on full oestrus and the result considered as positive when at least 50% of the animals used for the experiment showed non-nucleated pavement epithelium in the vaginal smear. In determining the threshold of activity and the duration of oestrus, a margin of error of about $\pm 15\%$ is to be taken into consideration.

Uterus growth test. The administration of the hormones was carried out in the same way as for the oestrus test. The increase and decrease of the weight of the uterus were controlled from time to time. The total dose used amounted generally to 50γ ($2 \times 25\gamma$). With both the free oestrone as well as its esters the uterine horns were usually filled to a marked degree with secretion which, however, sooner or later disappeared according to the duration of the effect. The presence of secretion may cause great variations of weight; the uterine horns were therefore cut open lengthways and carefully pressed out before weighing. All experiments were made on young rats.

It was first of all determined to what extent the weight of the uterus altered with the increase of body weight of the young animals without administration of hormone (Fig. 1). As will be seen from Fig. 1, the variations of the weight of the uterus are considerable; they can amount to $\pm 50\%$. A control of such results is always possible if the entire curve is considered. It was shown that with an increase of body weight from 30 to 80 g. the weight of the uterus increased on an average only about 30 mg., i.e. from 25 to 55 mg. However, in

heavier animals a rapid increase of weight occurs. With these animals it is apparently due to the fact that sexual maturity has set in. As younger animals still suck we did not use rats weighing less than 45 g. Animals weighing about 45 g. were of use for experiments lasting up to 30 days. It is interesting to

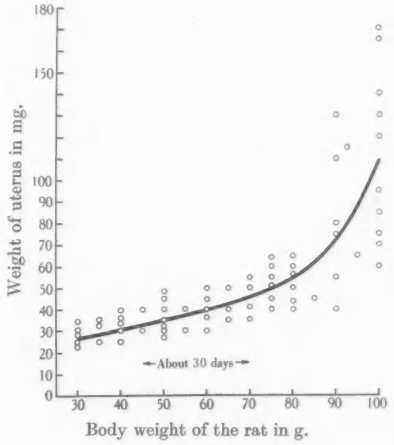

Fig. 1. Dependence of the weight of the uterus on the body weight of the normal rat.

observe that in determining the weight of the uterus of treated animals, the greater the increase of weight, the less is the margin of error, the margin of error being reducible to $\pm 10\%$.

The points of the following curves represent average values obtained on at least 3 rats; 450 rats were used in the experiments reported on in this paper.

(II) *The assay in the oestrus test*

Free oestrone. The average threshold value of oestrone, measured on the rat, amounts according to our method to $0{\cdot}7\gamma$; this is based on extensive tests. Values from $0{\cdot}35$ to $2{\cdot}5\gamma$ have been given by others but the difference in values is probably due to varying technique and also in part to different methods of assessing the results.

Activators. As with the male hormones the activity of oestrone can also be increased by the addition of so-called activators. Thus the duration of action of 50γ oestrone with the addition of 200 mg. of palmitic acid is 7 days and with the addition of 200 mg. stearyl alcohol it is 13 days, in contrast to 5 days when oestrone alone is used.

Esters. As is to be seen from Table I, the threshold value measured on the rat increases with aliphatic esters of oestrone according to the increase in chain length of the acid groups, first of all slowly up to the valerate ($0{\cdot}85$–$1{\cdot}5\gamma$), then more quickly up to the palmitate and stearate (up to 75γ). With the lower esters up to the valerate, oestrus appears after 3 or 4 days as is the case with free oestrone. With the higher esters up to the laurate, oestrus appears after 4 to 5 days and with palmitate and stearate only after 5 to 6 days.

Fig. 2 gives information on the duration of oestrus with various doses. It has been shown that with oestrone and its lower esters (acetate and propionate) even in high doses (50γ) the duration of oestrus amounts to not more than 5 days. Recently Parkes [1937], using the plumage test, has shown that the effect

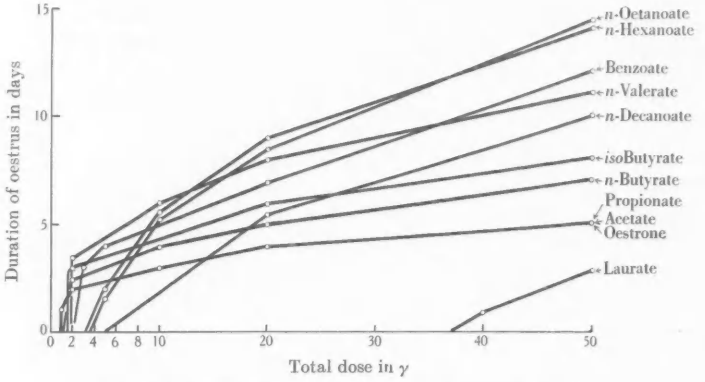

Fig. 2. The effect of oestrone and its esters on castrated rats in the oestrus test, using various doses.

of oestrone is hardly altered by acetylation. With the higher esters, however, the duration of oestrus is considerably prolonged and with *n*-hexanoate and *n*-octanoate in total doses of 50γ lasts for 14 days. Oestrone palmitate and oestrone stearate in total doses of 50γ show absolutely no effect. With doses of 100γ the duration of oestrus is about 30 and 40 days respectively.

The increased duration of effect of oestrone benzoate in the oestrus test was observed for the first time by Butenandt [1930] and also recently in the plumage test by Parkes [1937]. The activity of oestrone benzoate lies approximately between those of the valerate and the *n*-hexanoate.

(III) *The assay in the uterus growth test*

From Fig. 3[1] may be seen the relatively transitory and not very intense effect on the uterus of free oestrone when given subcutaneously in sesame oil. Acetate and propionate show slight increases of the duration of effect. From the butyrate onwards the increase is more pronounced. The most pronounced effect as regards intensity and duration is to be observed with *n*-octanoate.[2] With *n*-decanoate the intensity of effect begins to diminish. As in the oestrus test *iso*butyrate is more effective than *n*-butyrate. Also in this instance the activity of the benzoate is between those of valerate and *n*-hexanoate.

Surprisingly enough the maximal effects of both free oestrone and of its esters are attained always on the 4th day, i.e. about the time full oestrus sets in. In the uterus test the increase and decrease of effect can be followed much

[1] On account of simplicity an uterus weight of 50 mg. was uniformly selected as the starting point for the curves.

[2] It is possible that the maximum lies with the oenanthic acid ester.

better than in the oestrus test, e.g. an increase of the weight of the uterus can already be observed during pro-oestrus. Even after the termination of full oestrus the weight of the uterus during metoestrus diminishes only gradually.

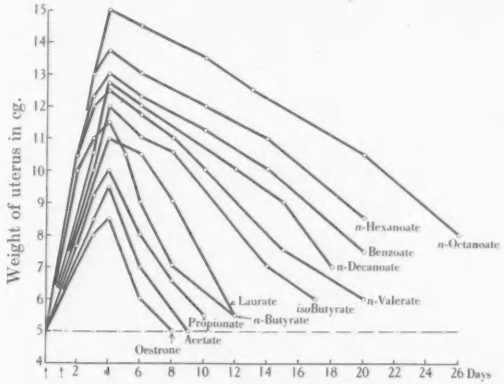

Fig. 3. The effect of oestrone and its esters on the weight of the uterus of young rats after administration of a total dose of 50 γ of hormone given in equal parts on two consecutive days.

(IV) *The evaluation of the quantitative measurements for the characterization of the effect of a compound*

If the compounds tested in this work were judged merely from their threshold values in the oestrus test, then absolutely false conclusions as regards their activity would be obtained. This was shown both by our experiments in the oestrus test and in the uterus test with higher doses of hormone.

Fig. 4 shows the changing course of the threshold value S_o (based on oestrone = 0·1 γ) in the oestrus test, of the maximal increase of weight $D_{max.}$ attained with each ester, as well as of the duration T of the total effect (partly estimated) in the uterus test in relation to the increasing chain length of the acid radical. $D_{max.}$, T and the day of the appearance of the onset of the maximum effect $t_{max.}$ give a satisfactory picture of the effect of a certain dose of a compound. This applies particularly if, as here, a pronounced maximum of the effect exists. However, a maximum effect does not always exist as will be shown later in the series of the oestradiol esters.

The integration of the curves showing the increase of weight gives a fairly clear idea of the total effect attained by a compound in the uterus test. Reference has already been made to this elsewhere [Miescher *et al.* 1937, 2; Miescher, 1937]. The integration is done most suitably by planimetric measurement of the area bounded by the curve representing the increase of weight and by the abscissa intercepting the starting point of the curve. The obtained output of activity (*Wirkungsausbeute*) A, possesses the dimension weight × time and can be expressed in the present case, e.g., in cg.-days so long as one measures the increase in weight in cg. and the time in days.[1] The unit of the output of activity is 1 cg.-day. It corresponds to an increase of weight of the uterus of 1 cg. in 1 day. We are now in a position to give the output of activity in an absolute

[1] The dimension would be volume × time, e.g. μl.-days, if the volume of the organ were measured and surface × time, e.g. mm.^{2}-days, if the surface (cock's comb) were measured.

measure; it is e.g. for oestrone 12·2 cg.-days; for the benzoate 144·4 cg.-days and for *n*-octanoate 185·4 cg.-days (that is 1·85 g.-days or 44·5 g.-hours).

The efficiency coefficient (*Nutzeffekt*) *c*, which is the quotient of the output of activity of oestrone A_o and its esters A_e, shows how many times more effective is an ester than the free hormone. As can be seen from Fig. 4 the efficiency coefficient of the benzoate is 9·3 and of the *n*-octanoate 15·2.

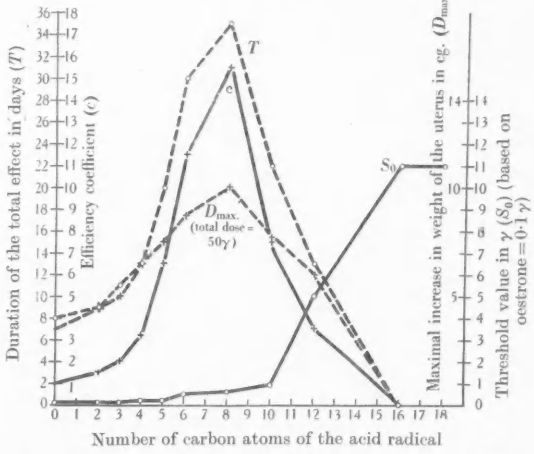
Duration of the total effect in days (T)
Efficiency coefficient (c)
Maximal increase in weight of the uterus in cg. (D max.)
Threshold value in γ (S0) (based on oestrone = 0·1 γ)
T
c
D max. (total dose = 50γ)
S0
Number of carbon atoms of the acid radical

Fig. 4. Influence of the chain length of the acid radical of oestrone esters on the maximal increase in weight of the uterus, on the duration of the total effect and on the efficiency coefficient in the uterus growth test as well as on the threshold value, based on oestrone = 0·1 γ, in the oestrus test.

If the weight of the uterus at the beginning of the test is designated as G_o and at the time of the maximum effect as $G_{\max.}$ then the maximum increase of weight is $D_{\max.} = G_{\max.} - G_o$ and the relative maximum increase of weight is

$$d_{\max.} = 100 . \frac{D_{\max.}}{G_o} \%.$$

Finally the mean increase of weight D_m during the total duration of the experiment is calculated from the equation

$$D_m = \frac{A}{T},$$

and from this the relative mean increase of weight can be obtained as follows:

$$d_m = 100 . \frac{D_m}{G_o} \%.$$

In Table II all the particulars obtained from the uterus test of the tested compounds are given.

We believe that our method of giving the figures under the various headings here in the table is of general importance for the characterization of the effect of any compound. Actually a corresponding method, but without the extension given here, was first used by Banting [1923, 1, 2] and later by Burger & Kohl [1933] and Kohl [1936] for the determination of the effect of insulin on the blood sugar.

Table II

Substance	Absolute values: A cg.-days	$D_{max.}$ cg.	$t_{max.}$	T days	D_m cg.	Relative values (Based on $G_o=5$ cg.): $d_{max.}$ %	d_m %	Relative values (Based on oestrone = 1): c
Oestrone	13·1	3·5	4	8	1·6	70	33	1·0
Oestrone acetate	18·4	4·5	4	9	2·0	90	41	1·4
Oestrone propionate	25·0	5·0	4	11	2·3	100	45	1·9
Oestrone *n*-butyrate	38·6	6·5	4	13	3·0	130	59	2·9
Oestrone *iso*butyrate	65·8	7·0	4	20*	3·3	140	66	5·0
Oestrone *n*-valerate	85·4	7·5	4	23*	3·7	150	74	6·5
Oestrone *n*-hexanoate	138·0	8·7	4	30*	4·6	174	92	10·5
Oestrone *n*-octanoate	184·8	10·0	4	35*	5·3	200	106	14·1
Oestrone *n*-decanoate	91·1	7·8	4	22*	4·1	156	83	6·9
Oestrone laurate	41·6	6·0	4	13*	3·2	120	64	3·2
Oestrone benzoate	112·4	8·0	4	26*	4·3	160	86	8·6

* Estimated.

Summary

1. The influence of the addition of "activators" such as palmitic acid and stearyl alcohol on the effect of oestrone in the oestrus test on the rat following subcutaneous injection was ascertained. Similar relations apply here as with the male hormones. Stearyl alcohol (200 mg.) increases the duration of effect of oestrone (50γ) from 5 to 13 days.

2. One aromatic and eleven aliphatic esters of oestrone were tested. They were subjected to a thorough investigation in order to ascertain their effects in the oestrus and uterus growth tests in the rat when given subcutaneously, in comparison with the effect of the free hormone.

3. *Oestrus test.* As regards the threshold value and the duration of oestrus, the acetate and propionate do not differ to any great extent from free oestrone. With the higher esters the threshold value increases first of all slowly up to the *n*-hexanoate ($S_o<1\gamma$) then, however, more quickly up to the stearate ($S_o=11\gamma$). From the butyrate upwards the duration of oestrus in comparison with the duration caused by free oestrone is increased. By using a total dose of 50γ the duration attains its maximum with *n*-hexanoate and *n*-octanoate (14 days). The benzoate is very near to valerate as regards effect. Palmitate and stearate show prolonged effects only in comparatively high doses.

4. *Uterus growth test.* The alteration of the weight of the uterus of normal rats during growth was controlled quantitatively. For experiments on young animals only rats weighing less than 80 g. can be used. The determination by curves of the time course of the weight of the uterus after administration of oestrone and its esters shows a particularly exact analysis of their effects. The sequence of the esters as regards their activity is approximately the same as in the oestrus test. Here oestrone *n*-octanoate proved to be the most active compound.

5. For the characterization of the effect of the investigated compounds on the uterus, the particulars of the maximal increase of weight, the day of the onset of effect and its total duration can be used. The maximum effect of oestrone and its esters is attained in every case on the 4th day. The total duration of effect on the uterus amounts approximately to twice that obtained in the oestrus test; as in the determination of the latter, pro-oestrus and metoestrus are not taken into consideration.

6. A particularly impressive comparison of the effects is obtained by ascertaining the absolute and relative outputs of activity (*Wirkungsausbeute*). The absolute output of activity in the uterus test is ascertained by planimetric measurement of the area bounded by the curve representing the increase of weight and the abscissa intercepting the starting point of the curve. The output of activity of oestrone amounts to 12·2, that of benzoate to 114·4, and that of *n*-octanoate to 185·4 cg.-days. The quotient from the output of activity of an ester and of the free hormone gives the efficiency coefficient (*Nutzeffekt*) attained by esterification (relative output of activity). It amounts for example with benzoate to 9·3 or with *n*-octanoate to 15·2.

7. The notions of the output of the activity and of the efficiency coefficient may, suitably adapted, be of general use in pharmacology.

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XXIII. COUPLED OXIDATION OF ASCORBIC ACID AND HAEMOCHROMOGENS

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By combined action of atmospheric oxygen and various reducing substances on haemochromogens green compounds are obtained which still contain iron, but less firmly bound than in haematin. Lemberg [1935] showed that these "green haemins" are bile pigment haemochromogens, closely related to biliverdins; they have been termed verdohaemochromogens.[1]

The process consists in oxidative cleavage of the porphyrin nucleus with removal of one of the α-methene groups, i.e. the same methene group as is removed in the biological process. When protoverdohaemochromogen is treated with acids biliverdin (dehydrobilirubin) results, and this substance is readily reduced to bilirubin by various tissue dehydrogenase systems [Lemberg & Wyndham, 1936]. A protoverdohaematin compound is therefore a possible intermediate substance between blood pigment and bile pigment.

A great variety of reducing substances appear to be able to induce verdohaemochromogen formation when pyridine-haemochromogen is shaken with air in their presence at about 50°, e.g. hydrazine, polyhydric phenols and, under certain conditions, SH compounds. Fischer & Lindner [1926] found extracts of yeast, minced plants or animal tissue (particularly liver) to be equally active; the reducing principle in these extracts was thermostable. That ascorbic acid acts rapidly in the same way even at room temperature was first observed by Karrer *et al.* [1933].

Barron, de Meio & Klemperer [1936] showed quite recently that ascorbic acid, when free from Cu and Fe, is not autoxidizable at $p\mathrm{H} < 8$, and that haemochromogens strongly catalyse the oxidation of ascorbic acid in neutral or slightly acid solutions. Simultaneous oxidative decomposition of the haemochromogens during this process was not noticed by these authors. If the results of Barron *et al.* and those of Karrer *et al.* are both correct, the oxidation of ascorbic acid and of haematin in pyridine is a coupled oxidation; neither ascorbic acid nor haematin alone undergoes oxidation under the conditions of the experiment.

It is important to investigate whether or not the coupled oxidation of haematin compounds and ascorbic acid is likely to occur under physiological conditions; and if so, how far it can account for haemoglobin breakdown in the animal body, and how far it can contribute to the destruction of vitamin C in the body. Secondly, it is desirable to study the mechanism of the coupled oxidation. In the present paper are described the technique with which we have studied the coupled oxidation of pyridine-haemochromogen and ascorbic acid quantitatively, and the results which we have obtained. Further evidence with regard to the mechanism of the reaction will be adduced in the second paper.

[1] Roche & Bénévent [1936] have recently suggested restriction of the use of the terms "haemin", "haematin" and "haemochromogen" to the iron complex salts of porphyrins. We do not find this suggestion practical and propose to use these terms in a wider sense, including the iron complex salts of all tetrapyrrolic compounds, of those with closed rings (porphyrins, chlorins, rhodins etc.) as well as of those with open chain (*iso*biliverdins, biliviolins).

Spectroscopic study of the reaction of pyridine-haematin with ascorbic acid

A dilute solution of haematin in 20% aqueous pyridine displays absorption bands at 565·2 and 530·7 mμ., both rather diffuse, the second band being the stronger. This spectrum is characteristic of ferric haemochromogen (pyridine-parahaematin). When 1 ml. of 1% ascorbic acid (B.D.H.) in water is added to a solution of 0·5 mg. haematin in 3 ml. 20% pyridine (prepared from crystalline haemin), the colour turns rapidly yellowish red and the typical absorption spectrum of ferrous pyridine-haemochromogen is seen. (Two absorption bands: I, 557·6; II, 525·2 mμ.) The first of the two bands is by far the stronger and both bands are stronger than those of the ferric haemochromogen. Therefore, the strength of absorption at about 557 mμ. of the ferric compound is negligible when compared with that of the ferrous compound, a fact which is of importance for some spectrocolorimetric determination methods used in this and the following paper.

If atmospheric O_2 is excluded, ascorbic acid causes no other alteration; when, however, the solution is shaken with air at room temperature (20°), it turns green in 2–3 min. The solution now displays the absorption spectrum of verdohaemochromogen: I, about 655; II, 531; III, 500 mμ. Intensities I (very strong); II, III. $Na_2S_2O_4$ causes the solution to turn yellow; band I persists, but bands II and III disappear and are replaced by general absorption in the blue; cautious shaking with air restores the green colour and the verdohaemochromogen spectrum. When HCl is added to the green solution, the colour is changed to blue-green and the absorption bands disappear and are replaced by absorption in the distant red end of the spectrum (biliverdin). The solution now gives the Gmelin reaction with nitric acid. These observations confirm Karrer *et al.* [1933] and show that ascorbic acid reduces ferric haemochromogen to ferrous haemochromogen, which undergoes oxidation to verdohaemochromogen when shaken with air in the presence of ascorbic acid. We can also confirm the observation of these authors that $Na_2S_2O_4$ protects haematin from oxidation to verdohaemochromogen, a fact of which we make use in our technique.

Non-autoxidizability of ascorbic acid in pyridine

We found that O_2 uptake and ascorbic acid destruction are negligible in a solution prepared by dissolving crystalline ascorbic acid (B.D.H.) in 20% pyridine (redistilled over KOH). The solutions had *p*H 6·9–7·9, varying with the amount of ascorbic acid.

A solution of 20·0 mg. of ascorbic acid in 1 ml. of distilled water was added to 9 ml. 20% pyridine. After 10 min. shaking in a Warburg manometer the solution contained the original amount of ascorbic acid, and after 30 min. only 0·03 mg. had been oxidized.

Technique

Reagents. Crystalline ascorbic acid (B.D.H.) was used throughout. Haemin (α-chlorohaemin) crystals were prepared from horse blood and ox blood in the usual way and recrystallized from pyridine-chloroform-glacial acetic acid. The pyridine was pure pyridine of Schering-Kahlbaum, redistilled over KOH.

Estimation of haematin

Principle of the method. For determination of total protohaematin in presence of verdohaemochromogen we have employed reduction with $Na_2S_2O_4$, followed by spectrocolorimetric comparison with a similarly reduced standard solution of

haematin. The first absorption band of haemochromogen at 557 mμ. is suitable for this purpose, since verdohaemochromogen does not absorb in this region and therefore does not interfere.

Since the absorption of ferric haemochromogen at 557 mμ. is negligible when compared with that of ferrous haemochromogen, the concentration of ferrous haemochromogen can be measured with sufficient accuracy even in presence of ferric haemochromogen. This allows approximate determination of the ratio of ferrous to ferric protohaemochromogens during the experiment.

. The apparatus consists of a Leitz Universal Colorimeter (with micro-cups and micro-plungers), on the top of which a Watson Microspectroscope was mounted. One micro-cup contains the solution to be examined, the other the standard solution. The field of vision shows the two spectra side by side. The thickness of the layer of the unknown solution is adjusted until the first absorption band is of the same strength as that given by the haemochromogen standard solution.

As standard a freshly prepared 100-fold dilution of a stock solution of 100 mg. haemin in 100 ml. 20% pyridine is reduced with $Na_2S_2O_4$ immediately before measurement. The stock solution keeps for weeks, but was not used for more than 1 week; the reduced standard remains unaltered for at least 30 min. It is essential to use pure pyridine. After some experience the readings can be taken with an accuracy of $\pm 3\%$.

The reaction under study is rapid and for accurate measurements speed in sampling and particularly in arresting the reaction is absolutely essential. We found that for the latter purpose $Na_2S_2O_4$ served well. A measured volume of the unknown solution was pipetted into a 5 ml. measuring flask containing some pyridine (20%) and a small knife-point of $Na_2S_2O_4$. The volume was then made up to 5 ml. with 20% pyridine, and the solution poured into one micro-cup of the colorimeter. The amount of the solution pipetted off was varied so that the ultimate haematin concentration was close to that of the standard. The spectro-colorimetric determination was done within 3 min. after sampling, but no appreciable change in haematin concentration was observed over much longer periods.

Estimation of ascorbic acid

After removal of haematin with trichloroacetic acid, ascorbic acid was usually titrated with $M/100$ iodine. Titration with 2:6-dichlorophenolindophenol gave quite similar results (see Table I). A small blank (0·06 ml. iodine per 15 ml. titrated solution) was subtracted. In some experiments in which glutathione was present the indicator method was used. A sharp end-point was always obtained unless the final ascorbic acid concentration was higher than 10 mg. per 100 ml., in this case the solution had to be diluted with 5% trichloroacetic acid before titration.

The ascorbic acid oxidation was arrested by precipitating the haematin by addition of 9 vol. 7% trichloroacetic acid to the 20% pyridine solution followed by filtration. Even so there was always some loss of ascorbic acid (Table I), which, with more haematin, was still more pronounced. Evidently the precipitated haematin still catalysed ascorbic acid oxidation. Additional oxidation was caused by ionized iron set free from verdohaemochromogen by the action of trichloroacetic acid. This could be prevented by addition to the trichloroacetic acid of cyanide sufficient to produce an ultimate concentration of $M/1000$. In agreement with other authors we found only 90–95% of the real ascorbic acid concentration in the trichloroacetic acid filtrate, even when haematin was absent. There

was, of course, some oxidation of ascorbic acid due to the almost instantaneous stoichiometric reaction between ferric haemochromogen and ascorbic acid. This could usually be neglected, however, since in most experiments a great molar excess of ascorbic acid is used. In the experiments of Table I the oxidation of ascorbic acid due to the stoichiometric reaction would be 0·02 mg. and thus within the limits of the error of estimation.

Table I

To 2 ml. of a solution containing 2·5 mg. ascorbic acid (and in the haematin experiments 0·31 mg. haematin) in 20% pyridine, 18 ml. of 7% trichloroacetic acid (containing 0·2 ml. *M*/10 NaCN in the cyanide experiments) were added immediately after the addition of the haematin. 10 ml. of the filtrate contained the following amounts of reduced ascorbic acid (in mg.):

	Trichloroacetic acid filtrates			
	Ascorbic acid present	Blank without haematin	With haematin	With haematin and cyanide
Iodine titration	1·24	1·14	0·92	1·15
Indicator titration	1·24	1·14	0·99	1·14

Reaction of ascorbic acid with pyridine-haematin in nitrogen

A solution of haematin in 20% pyridine contains ferric haemochromogen, which is quantitatively reduced to ferrous haemochromogen by excess of ascorbic acid. The first question to be considered is whether the oxidation of ascorbic acid by haematin is reversible or irreversible.

An aqueous solution of 1·27 mg. ascorbic acid was added under nitrogen to a solution of excess of haematin (25 mg.) in 20% pyridine. After 30 sec. 9 vol. of trichloroacetic-HCN mixture were added rapidly, and the haematin removed by filtration. The precipitate was washed several times with dilute trichloroacetic acid and one-half of the filtrate titrated with iodine directly, the other half after reduction with H_2S and removal of the H_2S by N_2. In the first half no ascorbic acid was found, in the second half the original amount of ascorbic acid had been fully restored by the treatment with hydrogen sulphide indicating that dehydroascorbic acid was the probable oxidation product. To investigate whether 1 or 2 mol. of haematin are required for the oxidation of 1 mol. ascorbic

Table II

Haematin γ	Ascorbic acid γ	Ratio ascorbic acid /haematin	Colorimeter reading	Haematin reduced γ	γ haematin which should be reduced provided the ratio be 1 : 2	γ haematin which should be reduced provided the ratio be 1 : 1	% of theoretical (1 : 2) reduction
			A. *Method* 1				
448	$Na_2S_2O_4$	—	43·0	448	—	—	100
224	120	2 : 1	18·9	197	224	224	88
448	120	2 : 2	33·1	345	445	448	77
224	60	1 : 1	16·3	170	222	224	76
224	60	1 : 1	15·6	162	222	224	72
448	60	1 : 2	30·4	316	222	445	70
224	30	0·5 : 1	16·6	173	111	222	77
448	30	0·5 : 2	14·0	146	111	222	65
			B. *Method* 2				
1·00	7·41	1 : 2	0·58	1·00	2·00	58	
2·00	7·41	1 : 1	0·80	1·00	2·00	80	
2·00	3·70	2 : 1	0·55	0·50	1·00	110	
2·00	1·76	2 : 0·5	0·29	0·24	0·48	120	

acid two methods were used. The excess of ascorbic acid remaining after reaction with a known amount of ferric haemochromogen was determined; secondly the concentrations of ferrous haemochromogen produced by insufficient amounts of ascorbic acid were measured.

Tables II A & B give the results, which show that 1 mol. of ascorbic acid reduces 2 mol. of haematin.

The coupled oxidation of ascorbic acid and pyridine-haemochromogen

Technique

The concentrations of ascorbic acid and haematin decrease during the coupled oxidation and, since the velocities of oxidation of both substances depend, as will be seen, on both concentrations, it is essential to measure the initial velocities in order to have clearly defined conditions.

The speed of the reaction is considerable, and cannot, as in other cases of haematin catalysis, be decreased by lowering the concentration of the catalyst, because it would then become impossible to make accurate haematin estimations. Particular care must be taken to ensure that the O_2 content of the solution is replaced with sufficient rapidity, so that the diffusion velocity of O_2 into the solution may not become a factor determining the speed of the reaction. Actually with low shaking velocities we found the speed of the oxidation of both haematin and ascorbic acid to depend on the velocity, particularly in the Warburg apparatus in which the horizontal shaking does not cause a sufficient contact of the liquid and gaseous phases. Rapid perpendicular manual shaking of about 3 ml. liquid phase in Thunberg tubes, however, gave satisfactory results. The rates of haematin and ascorbic acid oxidation under these conditions were considerably greater than in the experiments in Warburg flasks, particularly with high haematin and ascorbic acid concentrations (see Figs. 1–4) and were not increased by further acceleration of the shaking.

Thunberg tubes with bent, hollow stoppers were used throughout. They allow the reaction to be started suddenly by mixing the ascorbic acid solution in the stopper with the solution of haematin in dilute pyridine in the bottom part of the tube, and they also make it possible to work in various gas mixtures.

The tube was immersed in a thermostat to acquire the required temperature, the contents were mixed, the shaking begun at once and the stop-watch set going. The tubes were held by the side arms (so that the temperature equilibrium was not upset during the shaking) and they were shaken in such a way that the liquid remained in constant vigorous movement up and down the walls of the tube without splashing over into the hollow stopper. At the required time the shaking was stopped, the tube quickly opened and the samples of ascorbic acid and haematin measured out with pipettes. For each experiment a separate Thunberg tube was used. Usually ascorbic acid and haematin samples were taken from the same tube, one of us doing the experiment, the sampling and the ascorbic acid estimations, and the other the haematin estimations. Stopping the shaking retards the reaction velocity so much that no error is introduced in the few seconds which elapse between the taking of the two samples. No difference was found when the order of taking the samples was reversed. In experiments of short duration (up to 30 sec.) and with very small amounts separate operations for ascorbic acid and haematin were carried out; the total content of the tube was then available for one estimation. The curves showing the decrease of ascorbic acid and haematin concentrations with time are thus constituted of a

number of such separate experiments. Altogether more than 700 single experiments have been carried out, the reproducibility of the results being very satisfactory. Occasional stray values occurred, but in general, differences of the

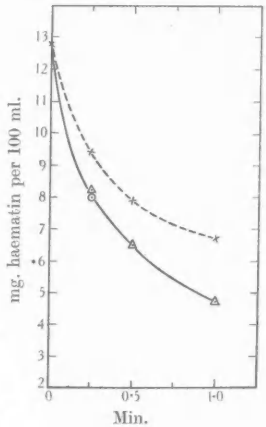

Fig. 1.

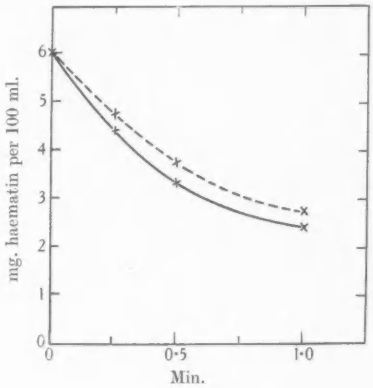

Fig. 2.

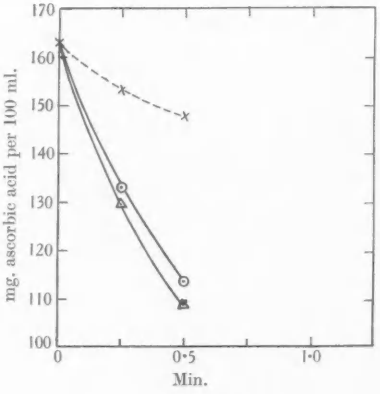

Fig. 3.

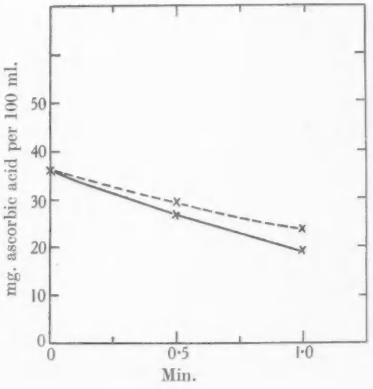

Fig. 4.

Figs. 1–4. Effect of shaking velocities on the rates of oxidation of haematin (Figs. 1 and 2) and of ascorbic acid (Figs. 3 and 4).

Figs. 1 and 3. 10 *mM*. ascorbic acid, 0·2 *mM*. haematin; Figs. 2 and 4, 2 *mM*. ascorbic acid, 0·1 *mM*. haematin.

—— Exps. in Thunberg tubes (shaking velocity 240 per min., ⊙; 350 per min., △).

– – – Exps. in Warburg flasks (shaking velocity 150 per min.; stroke, 2·5 cm.).

values for haematin and ascorbic acid concentrations between two separate experiments of the same kind did not exceed 10%, and the majority agreed within much closer limits. Each haematin or ascorbic acid value embodied in the curves is generally the average of two or three single values.

The products of the coupled oxidation

A solution containing 18·2 mg. ascorbic acid and 1·94 mg. haematin in 10 ml. 20% pyridine was shaken at 37° for 3 min.; 48·3% of the ascorbic acid was present in the reduced form and after reduction with H_2S 99·5% of the ascorbic acid was recovered. The oxidation of ascorbic acid during this time was therefore fully reversible. The product of the reversible oxidation is evidently rather stable under the conditions of our experiments. This is not in agreement with the observations of Borsook *et al.* [1937] that dehydroascorbic acid has only a very short life at neutral reaction; it is possible that pyridine stabilizes the lactone ring, the hydrolysis of which causes irreversible breakdown of dehydroascorbic acid. In the further course of the reaction there is some irreversible oxidation. After 20 min., when only 10% of the ascorbic acid was still in the reduced form and the haematin completely oxidized to verdohaematin, H_2S-reduction of the trichloroacetic acid filtrate restored only 64% of the original ascorbic acid.

It has been shown above that the product of the oxidation of pyridine-haemochromogen is verdohaemochromogen. The following experiment was carried out to see whether this substance is the only product of the reaction. 1·34 mg. haemin in 10 ml. 20% pyridine containing 30 mg. ascorbic acid were kept in the air for 5 hr. The solution was then green and protohaemochromogen had disappeared. A sample was diluted 12·5 times and compared with a similarly diluted standard solution of verdohaemochromogen [Lemberg, 1935] of the same molar concentration as the original protohaemochromogen solution. No difference was found in the strength of the bands in the red. From this it must be concluded that verdohaemochromogen is the only product of the reaction, at least when the reaction is carried out under mild conditions.

Measurements of the oxygen uptake

The oxidation of ascorbic acid to dehydroascorbic acid should require 0·5 mol. O_2, that of haemochromogen to verdohaemochromogen 1–2 mol. O_2 (according to whether the CH-group is removed as formaldehyde, as formic acid or as CO_2). In order to compare the actual O_2 uptake with that calculated from the destruction of ascorbic acid and of haemochromogen we have measured the O_2 uptake in Warburg manometers and determined in parallel experiments the destruction of ascorbic acid and haemochromogen occurring under the same conditions. The possibilities of such experiments are, however, limited. It will be seen below that the ratio of mol. ascorbic acid oxidized to mol. of haematin oxidized is always higher than 10. The O_2 uptake, due to the oxidation of haematin forms, therefore, only a small fraction of the total O_2 uptake and this makes it impossible to obtain conclusive evidence concerning the number of O_2 atoms required for the oxidation of haematin to verdohaematin. The evidence for the O_2 uptake of ascorbic acid, on the other hand, is conclusive, since the difference between O_2 uptake due to ascorbic acid and total uptake is small.

The experiments were carried out in Warburg manometers in flasks with a side bulb from which the ascorbic acid solution was tipped into the pyridine solution of haematin contained in the flask.

Table III shows that with initial concentrations of 0·2 *mM.* haematin and 9 *mM.* ascorbic acid the measured O_2 uptake is close to that calculated. With lower haematin and ascorbic acid concentrations however, particularly in the first minutes, the O_2 uptake is lower than it should be if the oxidation of ascorbic

acid involved one atom of O_2. These observations support the view of Bezssonoff & Woloszyn [1936; 1937] that under certain conditions a reversible oxidation product of ascorbic acid can be obtained which has only one H atom less than ascorbic acid. As yet we do not consider our results as conclusive evidence for the existence of this reversible oxidation stage of ascorbic acid, but they are certainly suggestive. This is further discussed in the following paper.

Table III

Initial haematin mg. in 3 ml.	Initial ascorbic acid mg. in in 3 ml.	Time of oxidation min.	$-\Delta$ haematin mg.	$-\Delta$ ascorbic acid mg.	$-\Delta O_2$ haematin calculated ($1O_2$/mol.) μl.	$-\Delta O_2$ ascorbic acid calculated ($\frac{1}{2}O_2$/mol.) μl.	$-\Delta O_2$ sum calculated μl.	$-\Delta O_2$ found μl.
0·38	4·80	0–0·5	0·15	0·36	5·1	22·9	28	32
0·18	1·08	0–3	0·135	0·84	4·6	53·6	58	52
0·10	0·96	2–3	—	0·220	About 1	14·0	15	6·3
0·10	0·96	3–5	—	0·236	About 0·5	15·0	15·5	11·5

The O_2 uptake continues almost undiminished after the complete oxidation of haematin (in Fig. 5 after 10 min.) and also after the total ascorbic acid has been oxidized (after 19 min.). After this time oxidation products of ascorbic acid must be further oxidized.

We have chosen the conditions of the experiment in Fig. 5 exactly as in the experiment given by Barron and coworkers to demonstrate the catalytic effect of pyridine-haemochromogen on the oxidation of ascorbic acid. The curve of

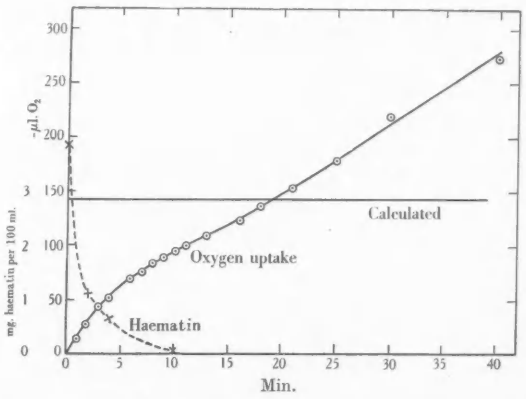

Fig. 5. Oxygen uptake in Warburg manometer. The horizontal line represents oxygen required for complete oxidation of ascorbic acid to dehydro-ascorbic acid and of haematin to verdo-haematin, the broken line haematin concentration.

0·096 mg. haemin and 2·4 mg. ascorbic acid in a total volume of 3 ml. containing 1·63 ml. of $M/10$ phosphate buffer pH 6·34 and 500 ml. pyridine. $T = 37°$. Shaking 150 oscillations per min.

these authors (over 240 min.) showed thus largely the effect of verdohaemochromogen on ascorbic acid and its further oxidation products. This also demonstrates how essential it is in our case to study the reaction in the initial stage in which interference by the oxidation products of both substances is still negligible.

Effect of complete oxidation of one component on the oxidation of the other

Determinations of the remaining ascorbic acid and of the remaining haematin also showed that the oxidation of the ascorbic acid proceeds, though with somewhat decreased speed, when all haematin has been oxidized to verdohaematin (Fig. 6).

We must conclude from these facts that verdohaemochromogen, like haemochromogen, has a catalytic effect on the oxidation of ascorbic acid. Verdohaemochromogen, in turn, is also oxidized in the further reaction, but much less rapidly than haemochromogen; in experiments with a great excess of ascorbic acid the solution ultimately turns brown and verdohaemochromogen is no longer present.

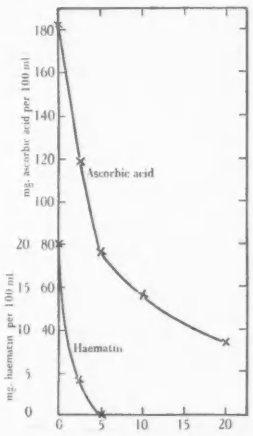

Fig. 6.

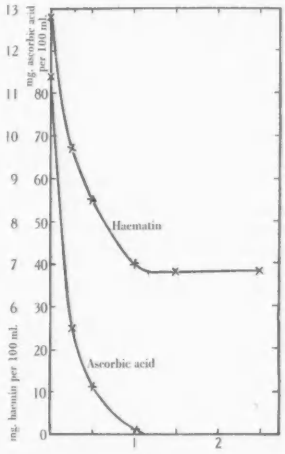

Fig. 7.

Fig. 6. Oxidation of ascorbic acid after complete oxidation of haematin. $T = 37°$.

Fig. 7. No oxidation of haematin after complete oxidation of ascorbic acid.

In experiments in which ascorbic acid became completely oxidized before the total haematin had been transformed into verdohaematin, the further oxidation of haematin ceased completely when ascorbic acid was no longer present, Fig. 7. At this moment the first absorption band of protohaemochromogen disappears and the remaining haematin is now in the ferric form.

Effect of various factors on the coupled oxidation. The oxidation ratio

It is to be expected that some factors might influence the rate of oxidation of one substance more than that of the other, so that the ratio of mol. ascorbic acid oxidized to mol. haematin oxidized will vary. We call this ratio the "oxidation ratio". The knowledge of its order of magnitude is evidently of interest in relation to the importance which a reaction of this type may have in the animal body.

The reaction velocity of the oxidation of either substance must depend on both haematin and ascorbic acid concentrations both of which undergo a constant change. A problem of this kind allows rigorous mathematical treatment only if

the oxidation velocity of one substance depends in a simple and constant manner on the concentration of the other, e.g. the mutual destruction of catalase and hydrogen peroxide. Here, however, no such simple relations exist, as will be seen below; this indicates that the reactions we are studying must be of a rather complicated nature, a supposition which will be supported by the evidence submitted in our second paper.

Effect of temperature. The influence of temperature variation on the rate of oxidation of haematin is shown in Fig. 8, and for ascorbic acid in Fig. 9.

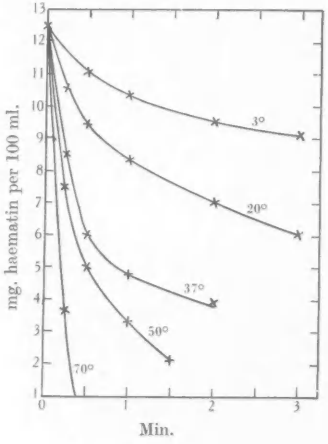

Fig. 8.

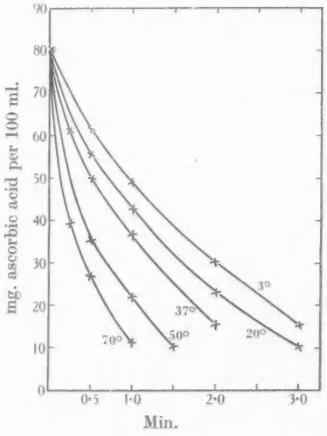

Fig. 9.

Fig. 8. Effect of temperature on oxidation of haematin.
Initial haematin concentration: ·02 *mM*. Initial ascorbic acid concentration: 4·5 *mM*.

Fig. 9. Effect of temperature on oxidation of vitamin.
Initial haematin concentration: 0·2 *mM*. Initial ascorbic acid concentration: 4·5 *mM*.

In Table IV the temperature coefficients of the initial rates of haematin and ascorbic acid oxidation are calculated. The initial velocities of the haematin oxidation have been taken from the curves graphically. For the velocities of the

Table IV

Temperature	Rate of haematin oxidation mg./100 ml. × min.	Q_{10} haematin	Rate of ascorbic acid oxidation k (unimol.) $= 1/t \log C_1/C_2$	Q_{10} ascorbic acid
3°	3·5	1·73	0·22	1·24
20°	8·9	1·46	0·32	1·20
37°	15·7	1·42	0·44	1·49
50°	24	1·23	0·74	1·21
70°	36		1·08	

ascorbic acid oxidation unimolecular reaction constants of ascorbic acid destruction have been used. At the first glance the latter procedure must seem wrong, since haematin catalyses the oxidation of ascorbic acid and the considerable alteration of the haematin concentration even in the early stages of the reaction should, therefore, not be neglected. It will be seen later, however, that the oxidation of ascorbic acid proceeds, indeed, rather closely according to the unimolecular law and that this apparently paradoxical fact is due to verdohaemochromogen, the oxidation product of haemochromogen, being also a catalyst of ascorbic acid oxidation, not very inferior to haemochromogen.

The average Q_{10} of ascorbic acid oxidation in all the experiments done at 20 and 37° was 1·30, that of haematin oxidation 1·86, somewhat higher than in the experiment shown in Table IV. From these data the apparent activation energies of haematin and ascorbic acid oxidations are 10,000 cals. for haematin oxidation and 4300 cals. for ascorbic acid oxidation. These values are given with the utmost reserve, particularly the activation energy for the haematin oxidation (cf. also the second paper). At higher temperatures Q_{10} haematin appears to decrease considerably.

From the experiments embodied in Figs. 8 and 9, the size of the oxidation ratio can be deducted. It is seen that the oxidation ratio decreases with increasing temperature; after 1 min. at 3° 59 mol. of ascorbic acid are destroyed per mol. haematin, at 37° only 20; a great molar excess of ascorbic acid is thus oxidized in the reaction.

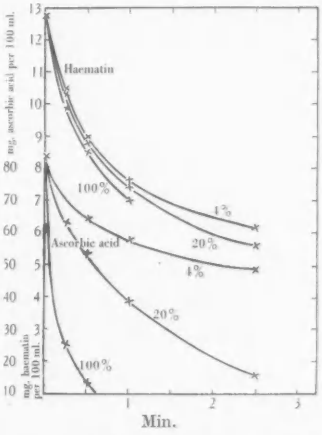

Fig. 10. Effect of oxygen pressure on the rate of oxidation of haematin and ascorbic acid. $T = 20°$

Effect of oxygen pressure. Of all factors the pO_2 has the most striking effect on the oxidation ratio. When 0·2 *mM.* haematin and 4·5 *mM.* ascorbic acid were oxidized in 20% pyridine at 20° the following oxidation ratios were found after 1 min.: in O_2: 59; in air: 32; in 4% O_2–96% N_2: 12. Fig. 10 shows that this is due to the fact that the haematin oxidation does not depend on the pO_2 in a range of 30–760 mm. mercury.

The rate of ascorbic acid oxidation, however, is proportional to the pO_2, as shown by the data of Table V.

Table V

% oxygen of gas mixture	k_{uni} of ascorbic acid oxidation	k_{uni} in % of k_{uni} in oxygen
100	1·71	100
20	0·34	19·9
4	0·087	5·1

Effect of p*H.* The range over which the influence of the *p*H on the reaction can be studied is limited by the insolubility of pyridine-haemochromogen at *p*H < 6·5 and by the autoxidizability of ascorbic acid in solutions having *p*H > 8. A solution of 8 mg. ascorbic acid and 1·3 mg. haematin in 10 ml. 20% pyridine has *p*H 7·61. In the experiments summarized in Fig. 11 the *p*H of the solution was altered by addition of small amounts of *N*/50 NaOH or 1% acetic acid, the concentrations of ascorbic acid, haematin and pyridine remaining the same; the

*p*H was measured with the double hydrogen electrode. In Fig. 11 the rate of oxidations of ascorbic acid and haematin (decrease of concentration of haematin in mg. per 100 ml. and of ascorbic acid in mg. per 10 ml. during 30 sec.) is plotted against the *p*H.

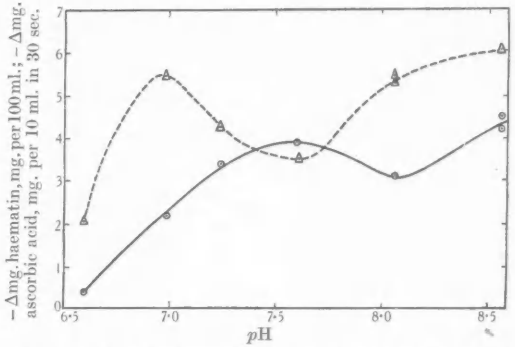

Fig. 11. Effect of *p*H on the rate of oxidation of haematin (solid line, ⊙) and ascorbic acid (broken line, ◬). Ascorbic acid: 80 mg.; haematin: 13 mg. per 100 ml.

The oxidations of ascorbic acid and of haematin are not affected by the *p*H in the same way. The oxidation of ascorbic acid reaches a maximum near neutrality; it falls off towards a minimum near *p*H 7·6 and then rises with increasing *p*H. The oxidation of ascorbic acid catalysed by haemochromogen thus depends on the *p*H in a way quite different from that of its autoxidation, which rises from about *p*H 8 towards the alkaline side.

The optimum *p*H for haematin oxidation is 7·6, distinctly more towards the alkaline side than that of the ascorbic acid oxidation; the velocity decreases somewhat to *p*H 8, but increases again above *p*H 8.

Effect of various initial concentrations of haematin and ascorbic acid. When the initial concentration of ascorbic acid is kept constant and that of haematin varied, the initial velocities of the oxidation both of haematin and ascorbic acid are roughly proportional to the initial concentration of the former (Figs. 12–14).

When the initial concentration of haematin is kept constant and that of ascorbic acid varied, the initial velocity of haematin oxidation increased with increasing ascorbic acid concentration, but not quite proportionally (Fig. 15). By varying the ascorbic acid concentration the *p*H is slightly altered, but this can be corrected by making use of the data of Fig. 11. After such correction the initial rate of haematin oxidation is roughly proportional to the initial ascorbic acid concentration in the range from 2 to 20 *mM*. ascorbic acid.

With equal initial concentrations of haematin the initial rate of oxidation of ascorbic acid is proportional to the initial concentration of the latter up to a concentration of about 9 *mM*. (Fig. 16); at higher concentrations the velocity of ascorbic acid oxidation decreased. The shift of *p*H caused by the variations in vitamin concentration does not greatly affect this result; in Table VI the decrease of ascorbic acid concentration during 30 sec. oxidation has been corrected for the *p*H shift, using the data of Fig. 11.

The decrease of the rate of oxidation with high ascorbic acid concentrations is thus real, and not caused by a *p*H shift. We have at present no explanation to offer for this phenomenon.

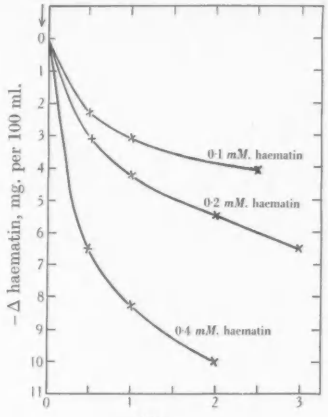

Fig. 12.

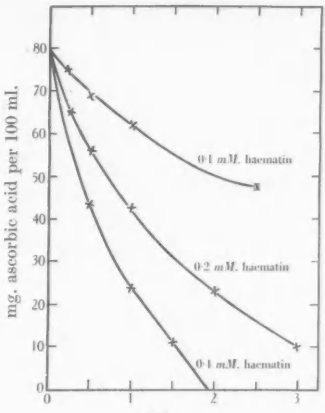

Fig. 13.

Fig. 12. Effect of initial haematin concentration on oxidation of haematin. Ascorbic acid: 4·5 *mM*. $T = 20°$.

Fig. 13. Effect of initial haematin concentration on oxidation of ascorbic acid. Ascorbic acid 4·5 *mM*. $T = 20°$.

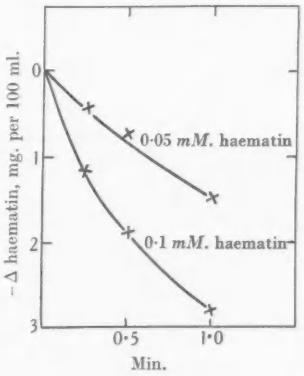

Fig. 14. Effect of initial haematin concentration on oxidation of haematin. Ascorbic acid: 1 *mM*. $T = 37°$.

Table VI

Initial ascorbic acid conc. *mM*.	*p*H	Correction factor from Fig. 11	– Δ mg./100 ml. ascorbic acid per 30 sec. Uncorr.	Corr.
2·2	7·89	0·75	16	12
4·5	7·61	1	27	27
9	7·25	0·82	51	42
18	6·99	0·64	39	25

From these observations it appears to be nearly correct that the velocities of both haematin and ascorbic acid oxidations are proportional to both haematin and ascorbic acid concentrations in the initial stage of the reaction. If they

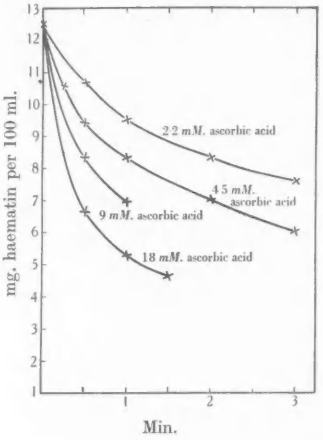

Fig. 15.

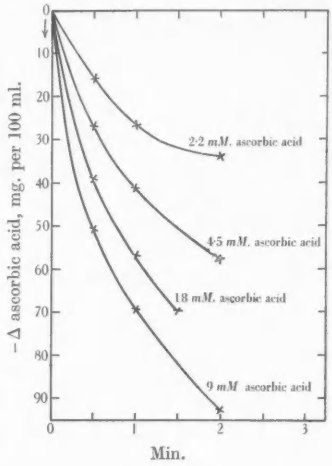

Fig. 16.

Fig. 15. Effect of initial ascorbic acid concentration on rate of haematin oxidation. Haematin: 0·2 *mM*. $T = 20°$

Fig. 16. Effect of initial ascorbic acid concentration on oxidation of ascorbic acid. Haematin: 0·2 *mM*. $T = 20°$.

remained so during the coupled oxidation, the mutual effect of pyridine-haemochromogen and ascorbic acid would be kinetically the same as the mutual effect of catalase and hydrogen peroxide, and we should be able to express the course of the concentration-time curves mathematically. The "unimolecular reaction constant" (mean reaction velocity) $k_h = \frac{1}{t_2 - t_1} \log \frac{h_1}{h_2}$, where h_1 and h_2 are the haematin concentrations at the times t_1 and t_2, ought then to be proportional to the mean concentration of ascorbic acid $v' = \frac{v_1 + v_2}{2}$; and correspondingly the unimolecular reaction constant of the ascorbic acid oxidation k_v should be proportional to the mean haematin concentration $\left(h' = \frac{h_1 + h_2}{2}\right)$. These laws do not however hold, as Table VII clearly shows.

Table VII

Time min.	$T = 3°$				$T = 20°$				$T = 37°$			
	k_h	$k_h/v' \times 10^3$	k_v	$k_v/h' \times 10^3$	k_h	$k_h/v' \times 10^3$	k_v	$k_v/h' \times 10^3$	k_h	$k_h/v' \times 10^3$	k_h	$k_v/h' \times 10^3$
0–0·25	0·130	1·73	0·23	19·3	0·286	3·95	0·36	31·2	0·67	9·46	0·46	43·5
0·25–0·5	0·092	1·37	0·225	19·9	0·209	3·60	0·26	25·9	0·60	10·9	0·38	52·5
0·5–1	0·057	1·07	0·20	18·5	0·108	2·19	0·24	27·2	0·21	4·9	0·26	49·1
1–2	0·035	0·89	0·21	21·5	0·074	1·74	0·27	34·8	0·07	2·7	0·39	88·8
2–3	0·019	0·82	0·29	30·8	0·067	4·16	0·36	54·7	—	—	—	—

k_h/v' and k_v/h' are not constant, k_h drops much more rapidly in the initial stages of the reaction than the mean concentration of ascorbic acid. Later, its decrease becomes less rapid, sometimes proportionally to the decrease of ascorbic acid concentration and sometimes even independently of it. That the later stages of the haematin oxidation do not depend on the ascorbic acid concentration becomes particularly clear by comparison of the two curves in Fig. 17, which show the decrease of haematin concentration with two different initial concentrations of ascorbic acid at 3°.

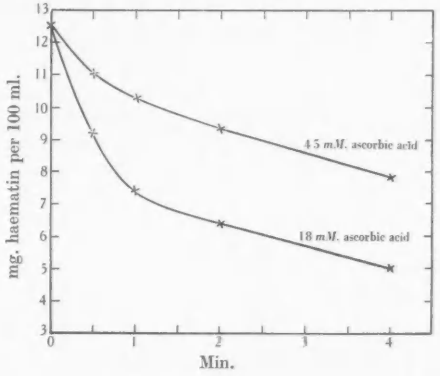

Fig. 17. Effect of initial ascorbic acid concentration on oxidation of haematin. Haematin: 0·2 mM. $T = 3°$.

Whereas the initial rates of haematin oxidation are proportional to the initial ascorbic acid concentrations, the later course of haematin oxidation is apparently independent.

These facts can only be explained by assuming that there are at least two reactions involved in the destruction of haematin: a rapid initial reaction, the rate of which depends on ascorbic acid concentration, and a slower reaction following in the later stages and being less dependent on or even independent of ascorbic acid concentration. In our second paper we shall present evidence for the existence of two different reactions which may account for these phenomena.

We have demonstrated above that the initial rate of ascorbic acid oxidation is proportional to the initial haematin concentration up to an ascorbic acid concentration of 9 mM. From Table VII, however, we see that k_v seems to remain definitely more constant, though with considerable variations, than k_v/h'; the reaction seems to be unimolecular and not dependent on haematin concentration. Here the correct explanation appears to be that when haematin is oxidized the catalytic action does not disappear but is replaced by that of verdohaematin which is also catalytically active though somewhat less so. A slight initial drop of k_v is indeed noticeable throughout. The tendency of k_v to increase again later is perhaps caused by a partial removal of iron from verdohaematin, producing iron catalysis of the vitamin oxidation.

We can now understand why the oxidation ratio varies somewhat with the time during which it is determined; it is lowest at first and rises later. Variations of the haematin concentration have little effect on the oxidation ratio; increase of the ascorbic acid concentration (up to 9 mM.) increases it a little in the initial stages and to a somewhat greater extent later.

Variation of the pyridine concentration between 5 and 20% of pyridine has no effect on the rate of oxidation of either haematin or ascorbic acid.

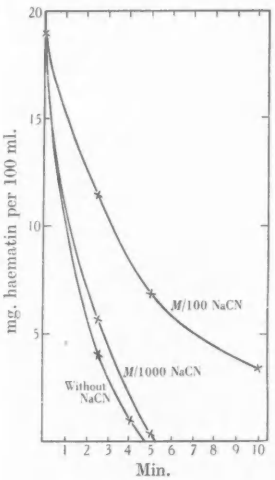

Fig. 18.

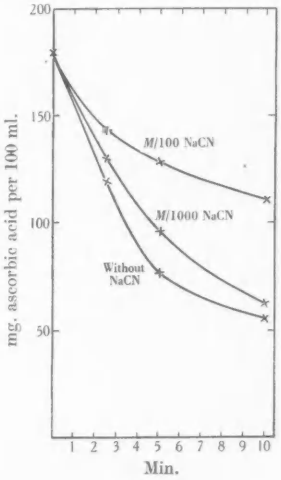

Fig. 19.

Fig. 18. Effect of cyanide on oxidation of haematin. Ascorbic acid: 10 *mM.*; haematin: 0·3 *mM.* $T = 30°$.

Fig. 19. Effect of cyanide on oxidation of ascorbic acid. Ascorbic acid: 10 *mM.*; haematin: 0·3 *mM.* $T = 20°$.

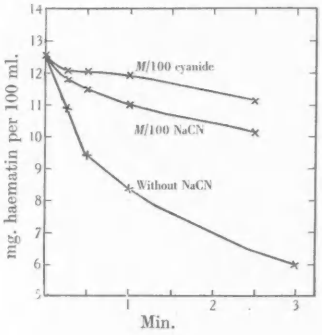

Fig. 20.

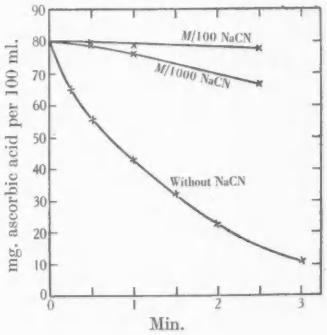

Fig. 21.

Fig. 20. Effect of cyanide on oxidation of haematin. Ascorbic acid: 4·5 *mM.*; haematin: 0·2 *mM.* $T = 20°$.

Fig. 21. Effect of cyanide on oxidation of ascorbic acid. Ascorbic acid: 4·5 *mM.*; haematin: 0·2 *mM.* $T = 20°$.

Effect of cyanide. With 10 *mM.* ascorbic acid and 0·3 *mM.* haematin *M*/1000 cyanide inhibits the oxidation of both haematin and ascorbic acid very little (about 10%), *M*/100 cyanide somewhat more (about 50%) and both

haematin and ascorbic acid oxidations in approximately the same proportion (Figs. 18 and 19). When the concentration of ascorbic acid is smaller, the inhibition by the same concentration of cyanide is considerably greater (Figs. 20 and 21).

The influence of the ascorbic acid concentration on the degree of cyanide inhibition is explained by the following experiment. Cyanide was added to a 0·4 *mM.* solution of haematin in 20 % pyridine to *M*/100 concentration. The two bands of pyridine-parahaematin had then disappeared and were replaced by a single diffuse band of pyridine-cyanhaematin. This solution was placed in the bottom part of Thunberg tubes, and various amounts of ascorbic acid solution were filled into the hollow stoppers. The tubes were then evacuated and filled with N_2, the contents mixed and the degree of reduction of pyridine-cyanhaematin to haemochromogen judged by the strength of the two absorption bands of haemochromogen which appeared. There was evidently complete or almost complete reduction when the ascorbic acid was 10 *mM.* When, however, the ascorbic acid concentration was only thrice the theoretical (0·6 *mM.*) the haemochromogen bands were just visible, showing that the reduction was very incomplete. When the tube with the larger amount of ascorbic acid was opened and shaken with air, the spectrum again displayed the pyridine-cyanhaematin band in addition to weaker haemochromogen bands and soon the verdohaemochromogen band in the red appeared. There is evidently an equilibrium between pyridine-cyanhaematin, pyridine-haemochromogen, cyanide and ascorbic acid, and the greater the concentration of vitamin, the less cyanide is bound to the haematin. A high concentration of ascorbic acid can thus counteract the influence of cyanide. It remains, however, to be explained why occasionally the ascorbic acid oxidation is inhibited more strongly by cyanide than the haematin oxidation.

Effect of reduced glutathione. Hopkins & Morgan [1936] have shown that reduced glutathione protects ascorbic acid from oxidation by "hexoxidase", an enzyme oxidizing ascorbic acid, found in *Brassica* by Szent-Györgyi [1931]. This has been confirmed quite recently by Borsook *et al.* [1937] who showed that dehydroascorbic acid is reduced rapidly by glutathione. It was therefore of interest to see whether glutathione can protect ascorbic acid from the catalytic oxidation by pyridine-haemochromogen, and, if so, whether the verdohaemochromogen formation was also inhibited by glutathione.

For these experiments we used the technique described above with the only difference that the trichloroacetic acid filtrate was divided in two equal parts, in one of which ascorbic acid was determined with 2:6-dichlorophenolindophenol, while in the second part the sum of glutathione and ascorbic acid was determined by iodine titration.

When 0·2 *mM.* haematin in 20 % pyridine containing 200 mg. of glutathione per 100 ml. (6·5 *mM.*) was shaken with air in a Thunberg tube at 37°, the haematin content decreased only from 12·8 to 9·5 mg. per 100 ml. in 10 min. Verdohaemochromogen was not detectable, and after reduction with $Na_2S_2O_4$ a weak band at 584 mμ. was seen in addition to the haemochromogen bands. There is also no considerable oxidation of glutathione under these conditions (2·6 mg. per 100 ml. in 10 min.).

When, however, glutathione is added to a solution of pyridine-haemochromogen containing some ascorbic acid, formation of verdohaemochromogen takes place.

In Table VIII an experiment with added glutathione is compared with a control experiment without glutathione. It is seen that glutathione somewhat

Table VIII

Haematin: 0·1 *mM.*; ascorbic acid: 2·3 *mM.*; glutathione: 7·1 *mM.* in 20% pyridine. $T = 37°$. Shaken in Thunberg tubes in air. Control without glutathione.

Time min.	$-\Delta$ haematin mg. per 100 ml.	Control: $-\Delta$ haematin mg. per 100 ml.	$-\Delta$ ascorbic acid mg. per 100 ml.	Control: $-\Delta$ ascorbic acid mg. per 100 ml.	$-\Delta$ glutathione mg. per 100 ml.	$-\Delta$ glutathione in terms of mg. per 100 ml. ascorbic acid	$-\Delta$ ascorbic acid saved mg. per 100 ml.
0–1	0·43	0·36	0·52	2·08	6·38	1·83	1·56
0–2	0·60	0·39	1·24	2·85	6·74	1·93	1·61

increases the rate of oxidation of haematin in presence of ascorbic acid, but decreases considerably the oxidation of ascorbic acid. A somewhat greater amount of glutathione is oxidized than corresponds to the ascorbic acid protected from oxidation. This experiment and several others which gave identical results prove that glutathione preserves the ascorbic acid by back-reduction of its oxidation product, and that ascorbic acid acts in this way as hydrogen carrier between haematin and glutathione. The protective effect of glutathione is certainly much more pronounced when the oxidation of ascorbic acid proceeds less rapidly than in our experiment or when glutathione is present in a greater molar quantity. The *p*H in this experiment was 7·5, and this proves that glutathione can protect ascorbic acid from oxidation by haemochromogens at physiological reactions.

Effect of the nitrogenous haemochromogen base. Barron *et al.* [1936 1, 2] have shown that other haemochromogens (nicotine-, pilocarpine-) also catalyse the oxidation of ascorbic acid, but that haematin in phosphate buffer does not.

Likewise the formation of verdohaemochromogen is not limited to pyridine-haemochromogen though other haemochromogens react less rapidly. Nicotine-haemochromogen which is the strongest catalyst for the oxidation of ascorbic acid is much less oxidized to verdohaemochromogen. With 4·5 *mM.* ascorbic acid and 0·2 *mM.* haematin at *p*H 7·7 and 37° nicotine-haemochromogen gives after 1 min. in air an oxidation ratio $\frac{\text{mol. ascorbic acid}}{\text{mol. haematin}}$ of 107, whereas that of pyridine-haemochromogen is 20. With pilocarpine-haemochromogen the oxidations of both ascorbic acid and haematin are slower.

Denatured globin-haemochromogen is very little reduced by ascorbic acid, and when it was shaken with air for 5 hr. in the presence of ascorbic acid, no formation of verdohaemochromogen took place. After 24 hr. at room temperature, however, a distinct absorption band in the red was found.

Discussion

In the present paper we have shown that ascorbic acid and haematin undergo coupled oxidation in dilute pyridine solutions. Pyridine-haemochromogen catalyses the reversible oxidation of ascorbic acid, which is not autoxidizable under these conditions, and ascorbic acid induces the oxidation of haematin to verdohaematin, a bile pigment iron complex.

The catalysed oxidation of ascorbic acid leads mainly to dehydroascorbic acid and in the first stages with low concentrations of ascorbic acid and haematin probably to a monodehydrogenation product. The rate of the reaction depends on the O_2 pressure. It has a low temperature coefficient and as in many catalysed reactions the activation energy is low. Ferric haemochromogen oxidizes ascorbic

acid rapidly to dehydroascorbic acid, and ferrous haemochromogen is autoxidizable and readily gives ferric haemochromogen. In these results there is nothing conflicting with the assumption that the valency change of haemochromogen catalyses the ascorbic acid oxidation. The same explanation has been given by Barron *et al.*

Cyanide, which blocks the action of other haematin enzymes even in low concentration by uniting with the ferric form giving a stable and inactive compound, is less effective as an inhibitor in our case. This might suggest that the mechanism of the haematin catalysis cannot be explained by a valency change, and that the haemochromogen remains in the ferrous state throughout the reaction. It has been shown, however, that ascorbic acid reduces pyridine-cyanhaematin in the presence of pyridine to ferrous haemochromogen, and the fact that the degree of cyanide inhibition depends on the ascorbic acid concentration provides further evidence that the low degree of cyanide inhibition must be explained in this way.

If the oxidation of ascorbic acid is due to the oscillations of the valency of haematin alone, it can be calculated that we have in our experiments a "turnover number" (number of complete cycles of valency change) of between 20 and 50 per min. in air, and about 50–100 in O_2; the turnover number of nicotine-haemochromogen is still greater (45 as compared with 25 with pyridine-haemochromogen under the same conditions).

The catalytic effect of haematin compounds on ascorbic acid oxidation has also direct physiological interest. Barron *et al.* [1936] recently adduced evidence that the hexoxidases of cabbage [Szent-Györgyi, 1931] and of Hubbard squash [Tauber *et al.* 1935] are of haemochromogen nature. $M/200$ cyanide inhibited the oxidation of ascorbic acid in the presence of enzymes from the juices of cabbage, yellow squash and watercress considerably, whereas typical inhibitors of copper catalysis (8-hydroxyquinoline; glutathione) had no effect. (In these experiments there was not sufficient glutathione to make the ascorbic acid-protecting effect of glutathione demonstrable.) In some fruits and other vegetables (apple, peach, lettuce) copper may be active in addition to haemochromogens. We have attempted to concentrate the enzyme of the Hubbard squash sufficiently to see the haemochromogen bands in the spectroscope, and have, indeed, observed weak bands in the green. The α-band was measured with some difficulty and its position fixed at about 560 mμ.

Haemochromogens might be thought to be unsuitable as catalysts of ascorbic acid oxidation, since they also undergo destruction during the process. There are, however, considerable differences in the stability of various haemochromogens in this reaction, some of them being only slowly attacked. It is also not impossible that these enzymes may be haemochromogens of bile pigment type; verdohaemochromogen, the product of the oxidation of protohaemochromogen, which has been shown above to possess considerable catalytic activity for ascorbic acid oxidation, is very stable under the conditions of the reaction. The occurrence of small amounts of verdohaemochromogen in the liver has been demonstrated by Lemberg & Wyndham [1936] and its occurrence in plants is quite likely.

How far haemochromogen catalysis may contribute to normal or pathologically increased ascorbic acid destruction in the animal body, cannot yet be estimated. The protective action of glutathione will, as a rule, probably suffice to counteract any ascorbic acid oxidation by haemochromogens which might occur. It seems, however, not quite unreasonable to assume that pathologically increased formation of haemochromogens or haemolysis with simultaneous

lowering of the protective glutathione mechanism may cause the increased requirement of ascorbic acid which recent investigations show to be a typical feature of many infections.

As to the catalytic action of ascorbic acid on the oxidation of haematin to bile pigment haematin there can be no doubt that this is an oxidation, and it may seem surprising that the rate of the reaction does not depend on the O_2 pressure within large limits, though some O_2 is, of course, necessary. The explanation must clearly be that only ferrous haemochromogen undergoes this oxidation. The reaction velocity might then be proportional to the O_2 pressure, but it will also be proportional to the concentration of ferrous haemochromogen and this will be inversely proportional to the O_2 pressure within certain limits. This explains also the necessity of reducing agents for the reaction. The temperature coefficient, though rather low, is still of the order of normal non-catalysed reactions, e.g. the destruction of catalase by hydrogen peroxide, and this indicates that the formation of verdohaemochromogen is due to autoxidation of the haemochromogen, the role of ascorbic acid being only that of a reducer.

Why then cannot all reducing agents cause verdohaemochromogen formation? For example $Na_2S_2O_4$ is quite unsuitable, although it reduces ferric to ferrous haemochromogen; when such a solution is shaken with air ferric haemochromogen and not verdohaemochromogen results. This may be explained by the assumption that each time only a small proportion of the molecules of ferrous haemochromogen undergoes oxidation to verdohaemochromogen, whilst the greater number yield ferric haemochromogen. When the turnover number (calculated from the amount of ascorbic acid oxidized per min. per mole haematin present) and the percentage of haematin oxidized during the same time are compared, our experiments show that with pyridine-haemochromogen at least 100 oxidation cycles are necessary for the complete oxidation of haematin to verdohaematin; each time 99% of the haemochromogen is oxidized to the ferric state and only 1% to verdohaematin. In the presence of $Na_2S_2O_4$, which undergoes a more rapid oxidation than ferrous haemochromogen, the oxidation of the latter substance practically begins only after the $Na_2S_2O_4$ has been completely oxidized, so that there are only a few oxidation-reduction cycles, forming practically no verdohaemochromogen. Ascorbic acid, however, does not react with oxygen directly, but only with ferric haemochromogen and this is therefore repeatedly reduced and oxidized. Although each time only a small part may form verdohaemochromogen the cumulative amount becomes considerable. We shall bring evidence in the second paper that the process is not specific for ascorbic acid, but can be produced by other reducing systems which fulfil the same conditions.

Nicotine-haemochromogen which, to judge from its effect on ascorbic acid, has a greater turnover number than pyridine-haemochromogen, is much less rapidly oxidized and requires about 210 repeated oxidation and reductions for complete transformation to verdohaematin, about four times as many as pyridine-haemochromogen. Considering the complicated nature of the process leading to the formation of verdohaemochromogen, there are various ways in which such a difference can be explained, so that the discussion will be deferred until more is known of the nature of this process.

From the evidence of results described in this paper we can make the statement that the transformation of a haematin compound into a bile pigment iron complex which readily loses its iron proceeds rapidly by the reaction which we have studied, at physiological temperature and under physiological con-

ditions. It is possible to advance this as a hypothesis which may help to explain the problem of physiological bile pigment formation.

The main part of bile pigment formation certainly takes place in the cells of the reticulo-endothelial system, which probably contain ascorbic acid and glutathione. This fact has to be kept in mind in discussing an objection which may be raised against our hypothesis. Our experiments have been carried out with pyridine-haemochromogen, and pyridine can hardly be considered as a physiological cell constituent in spite of the fact that nicotinamide has already been stated to be an important physiological substance. It has been shown that the reaction is not limited to pyridine-haemochromogen, but so far no haemochromogen has been found which reacts with reasonable rapidity and which is likely to occur in the cell. Haemoglobin itself reacts slowly, though in the same direction, with ascorbic acid. It might very well be that still another hydrogen carrier has to be inserted between haemoglobin and ascorbic acid to bridge the considerable gulf between the redox potentials of the two substances, and that this substance occurs in the reticulo-endothelial cells. There is still another possibility. It is suggestive that the redox potential of pyridine-haemochromogen ($E_0' = -0{\cdot}050$ at pH 9·15 according to Barron *et al.* [1936, 2]) stands midway between that of haemoglobin ($E_0' = +0{\cdot}15$) and of denatured globin-haemochromogen (cathaemoglobin) which is very little reduced by ascorbic acid and thus has a redox potential rather close to that of haematin itself ($E_0' = -0{\cdot}24$ at pH 9·16) which behaves similarly. It does not appear unreasonable to assume that the process of haemoglobin breakdown in the recitulo-endothelial cells is initiated by a denaturation of haemoglobin which does not lead to normal cathaemoglobin, but to a protein-haemochromogen of somewhat higher redox potential and a behaviour similar to that of pyridine-haemochromogen. Recent work has revealed the great importance of specific proteins for many biological processes, and evidence is available indicating that the denaturation of globin proceeds in stages. In support of this hypothesis it may be recalled that the verdohaemochromogen, found by Lemberg & Wyndham [1936] accompanying catalase in horse liver extracts, has as protein component not globin, but a protein of a different nature, similar to that to which haematin is bound in catalase; moreover bilirubin in serum is combined with a protein having the properties of serum albumin, and the chemical composition of globin [Pedersen & Waldenström, 1937].

Summary

Pyridine-haemochromogen and ascorbic acid undergo a coupled oxidation by atmospheric oxygen, the oxidation of 1 mg. of ascorbic acid at 37° being coupled with that of about 0·2 mg. haematin. The product of the haematin oxidation is verdohaematin, a bile pigment iron complex; the main product of the oxidation of ascorbic acid is dehydroascorbic acid.

Other haemochromogens and haemoglobin react in the same way, though less rapidly.

Temperature variations affect haematin oxidation more than ascorbic acid oxidation (Q_{10} haematin = 1·8, Q_{10} ascorbic acid = 1·3).

Variations of oxygen pressure from 4 to 100% of an atmosphere have little effect on haematin oxidation, whereas the rate of ascorbic acid oxidation is proportional to the oxygen tension.

The optimum pH is 7·0 for oxidation of ascorbic acid and 7·6 for that of haematin.

The initial velocities of haematin and ascorbic acid oxidations are proportional to the initial concentrations of haematin and (within certain limits) of ascorbic

acid. The rate of ascorbic acid oxidation decreases in the course of the reaction, however, less than it should according to these laws, owing to the catalytic activity of verdohaemochromogen, and the rate of haematin oxidation decreases initially more than it should, owing to the complex nature of this process.

The degree of cyanide inhibition depends on the ascorbic acid concentration.

Glutathione, which slightly accelerates verdohaemochromogen formation with ascorbic acid, though alone it does not cause it, saves ascorbic acid from oxidation by back-reduction of its oxidation product. Ascorbic acid and glutathione thus transport hydrogen from activated substrates to ferric haemochromogen.

The bearing of these observations on the theory of the mechanism of the reaction and on the problem, whether the physiological breakdown of haemoglobin and the bile pigment formation may proceed in a similar way, is discussed.

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During the initial stages of this research Mr R. A. Wyndham has collaborated with us, and during its final stages Mr W. H. Lockwood.

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XXIV. CHEMICAL MECHANISM OF THE OXIDATION OF PROTOHAEMATIN TO VERDOHAEMATIN

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There are four assumptions possible for the mechanism of the reaction described in the previous paper by which ferrous haemochromogen is oxidized to verdohaemochromogen.

(1) The ferric haematin iron may oxidize the porphyrin nucleus.

(2) A peroxide of ascorbic acid may cause this oxidation. Krebs [1936] has observed a coupled oxidation of haemoglobin or haemoglobin derivatives and amino-acids in the presence of amino-acid oxidase, and has tentatively suggested an amino-acid peroxide as the first intermediate step of the oxidative deamination.

(3) The autoxidation of ferrous haemochromogen may lead directly to verdohaemochromogen. This need not be discussed however since it would still remain to be explained why neither porphyrins nor ferric haematin compounds undergo the oxidation to bile pigment compounds.

(4) Hydrogen peroxide, formed during the autoxidation of ferrous haemochromogen or of ascorbic acid, may cause oxidative scission of the porphyrin ring.

McLeod & Gordon [1922] have shown that the formation of "green pigment" from haemoglobin by certain bacteria [Schottmüller, 1903, "viridans effect"] can be brought about under certain conditions by the action of hydrogen peroxide on heated blood agar; pneumococci can themselves produce hydrogen peroxide. Hart & Anderson [1933] were however unable to transform haemoglobin into green pigment with hydrogen peroxide, nor did they observe inhibition of pigment formation by catalase; they showed later [Anderson & Hart, 1934] that the same pigment is produced by the simultaneous action of oxygen and reducing systems (ascorbic acid, cysteine, dihydroxyacetone, glucose+glycine+ammonia) on haemoglobin. From their observations and from what has been said in our previous paper, there can be little doubt that this reaction is closely related to the coupled oxidation of pyridine-haemochromogen and ascorbic acid.

Possibility (1) can be safely excluded by the following experiment: 40 mg. $Na_2S_2O_4$ were dissolved in 100 ml. of O_2-free water and amounts varying from 0·1 to 2·5 ml. filled into the bent, hollow stoppers of Thunberg tubes. Into each tube were introduced 5 ml. of haematin solution in 20% pyridine (1 mg. haematin). The tubes were then evacuated, filled with O_2-free nitrogen, and their contents mixed. Spectroscopic observation showed that the tube with the largest amount of $Na_2S_2O_4$ (2·5 ml.) contained ferrous haemochromogen only, whereas in all the other tubes (with 0·8, 0·4, 0·2 and 0·1 ml. $Na_2S_2O_4$) the reduction was incomplete and varied from about 80 to about 10%. These tubes thus contained mixtures of ferric and ferrous haemochromogens, and if ferric

haematin iron were able to oxidize the porphyrin nucleus of ferrous haemochromogen, verdohaemochromogen formation should occur. No alteration and no formation of the absorption band in the red, characteristic of verdohaemochromogen, could be observed, even when the tubes were kept for a week.

A similar experiment was carried out with ascorbic acid instead of $Na_2S_2O_4$, with the same negative result.

(2) This theory, rather improbable in itself, can also be shown to be incorrect. Ascorbic acid, under the conditions in which pyridine-haemochromogen is oxidized, is not autoxidizable in absence of haematin. When ascorbic acid is shaken with air in dilute pyridine solution, no O_2 is taken up, and when haematin is now added, its oxidation to verdohaematin is not accelerated when compared with a solution of ascorbic acid in pyridine from which O_2 has previously been exhausted. There is also no trace of the formation of the intermediate product, described in a later section (which possesses an absorption band at 639 mμ.), when a pre-shaken solution of ascorbic acid in dilute pyridine is mixed under N_2 with a solution of haematin in pyridine.

Still more conclusive is the observation, discussed in greater detail in a later section, that verdohaemochromogen is also formed when ascorbic acid is replaced by cysteine and Cu, whereas pure cysteine is not active. It is very unlikely that a cysteine peroxide should be formed only in the presence of Cu and cause the oxidation of haematin to verdohaematin.

(4) If hydrogen peroxide were to arise during the coupled oxidation of haemochromogen and ascorbic acid and cause the oxidative cleavage of the porphyrin nucleus, it should be possible to inhibit the oxidation of haematin by catalase. Catalase inhibits, indeed, the oxidation of haematin, though by no means completely, whereas it is without effect on the oxidation of ascorbic acid (Fig. 1).

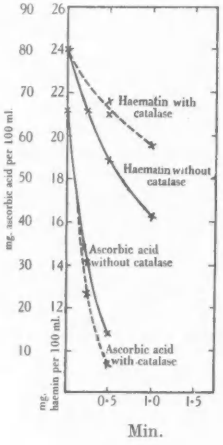


Fig. 1.

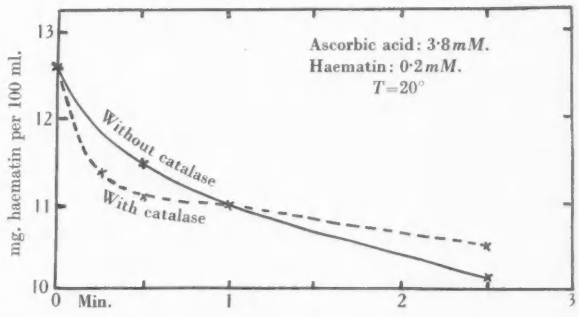


Fig. 2.

Fig. 1. Effect of catalase on the rate of oxidation of haematin and ascorbic acid. Haematin: 0·4 mM.; ascorbic acid: 3·8 mM.; $T=37°$. In experiments with catalase: 0·5 ml. catalase solution ($k=6000$) in 3 ml.

Fig. 2. Effect of catalase on the rate of haematin oxidation in the presence of $M/1000$ cyanide.

The last-mentioned fact makes it improbable that the inhibition of the haematin oxidation is caused by a non-specific effect of the catalase solution, e.g. a protein effect, since this would probably affect the oxidations of haematin and ascorbic acid to the same extent.

It can be shown, moreover, that $M/1000$ cyanide completely prevents the effect of catalase on the velocity of haematin oxidation (Fig. 2).

Since hydrogen peroxide is the only substrate upon which catalase acts rapidly, these observations make it very probable that hydrogen peroxide plays a part in the oxidation of haematin to verdohaematin.

Effect of hydrogen peroxide on pyridine-haemochromogen in the presence of ascorbic acid

Attempts to oxidize haematin in pyridine solution by H_2O_2, BaO_2 or CeO_3 in various concentrations were without success. With small amounts of peroxides haematin remained unaltered, the only alteration being that BaO_2 caused the solution to become alkaline and transformed ferric haemochromogen into alkaline haematin, and that H_2O_2 was destroyed catalytically. With larger amounts of peroxide a gradual decoloration took place. No verdohaemochromogen could be detected.

Attempts to oxidize pyridine haemochromogen by H_2O_2 in the presence of ascorbic acid seemed at first to give equally negative results. These experiments have, of course, to be carried out in nitrogen, since in the presence of oxygen verdohaemochromogen would be formed without H_2O_2.

Varying amounts of haematin in phosphate buffer *p*H 7·6, and of ascorbic acid, with a little pyridine were mixed in Thunberg tubes under N_2 with H_2O_2 (0·5–4 *mM*.). The tubes were then not examined until the following morning, when the apparent spectrum of protohaemochromogen was still strong, and in trichloroacetic acid filtrates H_2O_2 was detected by the titanic sulphate test. The conclusion from this that there had been no oxidation by H_2O_2 was invalidated by our finding that titanic sulphate gives a yellow colour with ascorbic acid, a reaction described by Ettori [1936]. The strength of the apparent haemochromogen absorption bands will be explained below.

When the experiments were repeated with BaO_2 we observed a distinct and rapid reaction, and the same could also be achieved with suitable amounts of H_2O_2. The red solution, containing ferrous pyridine-haemochromogen produced by action of ascorbic acid on ferric haemochromogen, turned brownish green on mixing with the dilute H_2O_2 solution or a few mg. of BaO_2 contained in the stoppers. A strong absorption band with the centre at 639 mμ. appeared which under suitably chosen conditions became the only band noticeable. A considerable excess of ascorbic acid was necessary. Complete disappearance of the haemochromogen and maximum formation of the band at 639 mμ. were produced when the following amounts of reagents, dissolved in 5 ml. dilute pyridine, were used: 0·4 mg. haemin, 0·5 mg. H_2O_2 and 12 mg. ascorbic acid. The molar ratio is ascorbic acid = 4·6 H_2O_2 = 113 haematin. When an excess of H_2O_2 or of ferricyanide was added after the green compound had been produced, the solution turned brown and displayed no visible absorption bands. These additions were made through the side-arms of the evacuated tubes under N_2.

The compound giving the absorption band at 639 mμ. could not be acid haematin, for in the presence of pyridine the latter was only formed when the *p*H was decreased to less than 4·5 with acetic acid, whereas the *p*H in our experiments was 7·9 with H_2O_2 and still higher with BaO_2. It was also not

verdohaemochromogen or any other haemochromogen,[1] for the absorption band at 639 mμ. disappeared on reduction with $Na_2S_2O_4$, the solution turning red again and strong haemochromogen bands reappearing apparently unaltered in position. The same occurred, though much more slowly, when the tubes were kept without adding reducer after the production of the green compound; in the course of a few hours the solution turned red again, the band at 639 mμ. disappeared and the haemochromogen bands returned. This explains why we had overlooked the reaction in our first experiments in which the tubes were not investigated at once.

The reappearance of the haemochromogen bands could be accelerated by the addition of catalase to the solution after the green compound had been produced as above.

When the tubes containing the green compound with the band at 639 mμ. were opened and shaken with air for a few minutes, the solutions turned pure green. The main absorption band was now found shifted to 652 mμ., the two other absorption bands of verdohaemochromogen being also visible, and $Na_2S_2O_4$ no longer destroyed the band in the red, but caused the solution to turn yellow. We must conclude from these experiments that the compound with the absorption band at 639 mμ., arising by the action of H_2O_2 on protohaemochromogen in the presence of ascorbic acid, is rapidly transformed into verdohaemochromogen by molecular O_2, but not by H_2O_2. The unexpected result of these experiments is that, though H_2O_2 may be essential for the reaction, it cannot replace molecular O_2 altogether.

The compound with the absorption band at 639 mμ. is formed as an intermediate product during the oxidation of haemochromogen to verdohaemochromogen by molecular oxygen

When the absorption band in the red part of the spectrum appears in this process, it is found to lie close to 640 mμ. and only gradually moves towards the red until it reaches the position at 652 mμ. When $Na_2S_2O_4$ is added in the initial stages, the band in the red disappears completely or becomes much weaker, whereas later it is intensified. These differences are particularly noticeable under conditions in which the reaction proceeds more slowly, for example, when it is carried out under diminished O_2 pressure. Cyanide does not prevent the formation of this compound by H_2O_2 or as intermediate product in the aerobic haemochromogen oxidation. It has also been found that addition of a small amount of BaO_2 or H_2O_2 accelerates haematin oxidation when haematin in pyridine is shaken with ascorbic acid in air.

Nature of the compound with absorption band at 639 mμ.

The situation of the two absorption bands of this haemochromogen were, within the limits of experimental error, the same as those of protohaemochromogen (557·4 and 526·3 mμ.; protohaemochromogen: 556·6 and 524·2 mμ.). The relative strengths of the two bands were different, however, the second band being distinctly broader and at least as strong as the first, whereas the first band of protohaemochromogen is by far the stronger.

A quantitative spectrocolorimetric estimation confirmed the results of spectroscopic observation. In a Thunberg tube haematin in the presence of excess ascorbic acid was oxidized by H_2O_2 to the compound with 639 mμ. band. This was then reduced by adding $Na_2S_2O_4$ under nitrogen. The strength of both bands

[1] Biliviolinohaemochromogens have an absorption band in this region [Lemberg & Wyndham, 1936].

was determined in a layer of the solution in the tube by spectrocolorimetric comparison with standard haemochromogen. The control giving the strength of bands of protohaemochromogen was prepared in a Thunberg tube of the same dimensions, H_2O_2 being replaced by the same volume of H_2O.

In another experiment the reduction was carried out by keeping overnight with excess ascorbic acid.

This compound is formed by the action of H_2O_2 on pyridine haemochromogen in the presence of excess ascorbic acid. It is destroyed by excess of H_2O_2 or $H_3Fe(CN)_6$, and on reduction gives a compound with absorption bands in the position of those of protohaemochromogen. On this evidence it was reported by us [1937, 1] that the substance was a ferrous haem-H_2O_2 compound.

The absorption spectrum of the substance was unlike that of known haem-H_2O_2 compounds. Several have been described [Keilin & Hartree, 1935, 1; 1936; Keilin & Mann, 1937] but all have a 2-banded spectrum totally different from that of our compound. The reactions of our compound did not accord with those described in the above literature.

It was not until we discovered that the absorption spectrum obtained on reducing the green compound with the band at 639mμ. was not that of protohaemochromogen that we were able to discard our first view of its structure.

Table I

	Reading of haemochromogen standard	
	Absorption band I 557 mμ.	Absorption band II 525 mμ.
Control (without H_2O_2)	33	33
New haemochromogen after $Na_2S_2O_4$ reduction	12	27
New haemochromogen after reduction by ascorbic acid	12	38
New haemochromogen after reduction by ascorbic acid, $Na_2S_2O_4$ added	14	38

Table I shows that only 40% of the original strength of the first haemochromogen band can be restored after the treatment with H_2O_2, whereas the strength of the second band has hardly changed. The reduction by keeping overnight leads to the same result, showing that the reduction by the excess of ascorbic acid is slow, but complete. When the reduction is carried out in this way, the second band is somewhat stronger than in the control or after reduction by $Na_2S_2O_4$.

The haemochromogen recovered after H_2O_2 treatment was altogether different from protohaemochromogen. When the latter is shaken with air in the presence of ascorbic acid, it turns gradually green; when, however, a solution of the new haemochromogen was shaken with air, it turned green in a flash. The green solution contained at first the compound with the absorption band at 639mμ., later verdohaemochromogen. Hence the compound with the band 639mμ. is to be considered as the ferric haemochromogen of the pseudo-protohaemochromogen.

When a solution of pyridine-haemochromogen containing $Na_2S_2O_4$ and ascorbic acid is extracted with ether and glacial acetic acid, the haemochromogen passes largely unaltered into the ether, and can be extracted from it by Na_2CO_3. When, however, the solution of the pseudo-protohaemochromogen was treated in the same way, the ether solution was green, not red and the Na_2CO_3 solution contained verdohaematin instead of protohaematin.

Finally, in the presence of reducing substances protohaemochromogen is split by HCl to protoporphyrin. Under the same conditions the haemochromogen obtained by ascorbic acid reduction after hydrogen peroxide treatment yields neither porphyrin nor bile pigment, but a chlorin-like compound for which we suggest the constitution of an *oxyporphyrin*. We shall, therefore, call the new haemochromogen provisionally "oxyhaemochromogen" and the compound with the absorption band at 639 mμ. "oxyhaematin", though it might be ferric oxyhaemochromogen. The name "oxyhaematin" might cause confusion, since this name is used in other languages for hydroxyhaematin. The term oxyporphyrin-haematin is clumsy, but should be used where a mistake is possible. In a preliminary publication [1937, 2] we have already given a short report on these results.

These facts enable us to understand better some difficulties we had encountered in explaining the kinetics of the haematin oxidation, described in the last paper. The decrease in haematin concentration has been measured there by the decrease in strength of the first absorption band of haemochromogen. When oxyhaemochromogen is present, as it is during the initial stages of the reaction, not only the transformation of haemochromogen into verdohaemochromogen, but in addition 60 % of the transformation of haemochromogen into oxyhaemochromogen is measured in this way, since the strength of the first band of oxyhaemochromogen is only 40 % of that of haemochromogen. The actual destruction of haematin during the initial phase of the reaction is therefore still greater than it appears from these experiments, which had already given an unexpectedly high initial rate of haematin oxidation. We had already concluded that more than one process must be at work, and can now explain the phenomenon by assuming a rapid formation of oxyhaemochromogen from haemochromogen (with loss of 60 % of the extinction at 557 mμ.), followed by a somewhat slower oxidation of oxyhaemochromogen to verdohaemochromogen (with loss of the remaining 40 % of the extinction at 557 mμ.). We can also understand why the later part of the reaction does not depend on the vitamin concentration, whereas the initial reaction does. H_2O_2 arises only by the action of O_2 on ferrous haemochromogen, and also acts only on ferrous haemochromogen to give oxyhaematin, so that the action of ascorbic acid is required. Oxyhaematin, however, reacts with O_2 to give verdohaematin whether in the ferrous or in the ferric form, and ascorbic acid is therefore not required.

Preparation and properties of the oxyporphyrin

In each of ten Thunberg tubes the oxyhaematin was prepared under nitrogen as described above, and the oxyhaemochromogen obtained by keeping overnight. Through the side-arm of each tube 3 ml. of conc. HCl were sucked in under nitrogen, and the tubes were kept for 48 hr. They were then opened and the green solution filtered from a small amount of precipitate. The filtrate showed the following absorption spectrum: I, 621·5; II, 567 mμ. Intensities: I, II. There was also a very weak band in the green, and perhaps a further band in the extreme red.

With sodium acetate the pigment was brought into ether, in which it dissolved giving a blue-green colour. The spectrum showed four bands: I, 650; II, 584; III, 546; IV, 511 mμ. Intensities: I, IV, III, II. The spectrum is thus more like that of a rhodin than of a chlorin. The compound was completely extracted from ether by 1 % HCl, its Willstätter number being 0·25. In 20 % HCl the blue-green solution (brown in thick layer) showed two absorption bands (623·5 and 568·4 mμ.).

The Cu complex salt was obtained by heating the residue of the ethereal solution for a short time with alcoholic Cu acetate. Its solution displayed two bands: 573·5 and 537·5 mμ., the second being the stronger. When this absorption spectrum is compared with that of the protoporphyrin Cu complex (527·8 and 534·4 mμ., the first being the stronger) the same similarity is noticed as between the haemochromogens which we described in the previous section. This proves that the splitting of the haemochromogen with acid does not involve an alteration in the metal-free pyrrolic compound.

The properties of the complex salts show that our oxyporphyrin is different from otherwise very similar compounds obtained by Fischer and co-workers by the action of H_2O_2 on porphyrins in strong H_2SO_4 [Fischer *et al.* 1927; Fischer *et al.* 1930]. These compounds contain one or two oxygen atoms more than the corresponding porphyrins which Fischer assumes to be either on the methene groups linking the pyrrole rings or in the β-position of a hydrogenated pyrrole nucleus as in certain oxychlorins [Fischer & Lautsch, 1937]. Both, mono- and di-oxymesoporphyrin, are reported to have the same absorption spectrum, and Table II shows that this spectrum is indeed similar to that of our compound in neutral as well as in acid solutions. The difference in the absorption spectra between our compound and green porphyrin ozonides [Fischer & Deželic, 1934] is much greater. Our oxyporphyrin is, however, a much stronger base than the oxyporphyrins of Fischer (see the HCl numbers in Table II) and, while these give complex salts of the chlorin-type, our oxyporphyrin gives complex salts of the porphyrin type with the only difference that the ratio of the strengths of the two absorption bands is reversed.

Table II. *Absorption spectra of oxyporphyrin and related compounds*

	Absorption bands in mμ.				
	In ether-acetic acid	In 20% HCl	Cu salt	Pyridine haemochromogen	HCl number
Our oxyporphyrin	650, 584, 546, 511 (I, IV, III, II)	623·5, 568·5 (I, II)	573·5, 537·5 (II, I)	557, 527 (II, I)	0·25
Fischer's oxymesoporphyrin	642, (629), (613), 584, 540, 504 (I, III, IV, II)	621·5, 576, 537 (I, II, III)	620, 570, (513) (I, II)	Indistinct	14·5
iso-Aetioporphyrin ozonide	645, (614), 591, 524, 491 (I, IV, III, II)	—	—	—	—
Protoporphyrin	632, 579, 537, 503 (IV, III, II, I)	606, 558 (II, I)	573, 534 (I, II)	557, 524 (I, II)	—

A porphyrin which yields a haemochromogen with a strong second absorption band is phylloporphyrin, which carries a methyl group on one methene group of the porphyrin nucleus [Treibs & Wiedemann, 1928]. Although our oxyporphyrin has not yet been obtained in amounts sufficient for analysis, our observations make it probable that it carries a hydroxyl group on the methene group α which is eliminated when the porphyrin ring of oxyhaemochromogen is split open by oxygen with formation of verdohaemochromogen. In the complex salts this oxyporphyrin is in the form of hydroxyporphyrin, since the spectra are similar to those of porphyrins and particularly to phylloporphyrin in which the methene group also carries a substituent. In the free state the oxyporphyrin is probably present in the keto-form and the chain of conjugated double bonds of the porphyrin nucleus is thus interrupted, so that the spectrum is no longer that of a porphyrin but similar to that of chlorins or rhodins.

Experiments with cysteine

When a solution of metal-free cysteine hydrochloride is added to a solution of haematin in phosphate buffer *p*H 7·6, a peculiar type of absorption spectrum is formed. There is a strong band in the red (656 mμ.) and a weaker band at 551 mμ. This cannot be a verdohaematin compound, for on addition of pyridine the band in the red disappears completely and pyridine-haemochromogen is formed. This observation shows that haematin probably forms a compound with cysteine having an absorption band in the red. Haemoglobin gives a similar reaction and a compound with an absorption band at 653 mμ. and two weak bands in the green is formed; this spectrum persists when the solution is shaken with air or when $Na_2S_2O_4$ is added, but it is immediately destroyed by pyridine. We shall investigate these reactions further but they have no direct bearing on the problem discussed here.

The presence of pure cysteine (Schering-Kahlbaum, freed from traces of metal according to Warburg) does not prevent the formation of verdohaemochromogen when protohaemochromogen is shaken with ascorbic acid in air; the initial formation of oxyhaematin also occurs as indicated by the appearance of the absorption band at 639 mμ. in the first stages of the reaction.

Pure cysteine alone does not, however, reduce pyridine-haematin to haemochromogen, and when the mixture is shaken with air, no verdohaemochromogen is formed. On reduction with $Na_2S_2O_4$ there is seen a weak absorption band at 584 mμ. besides those of protohaemochromogen. It has been reported in the preceding paper that glutathione acts in the same way. These observations are of special interest, since they show a way in which cytochrome-*a*-haematin may be formed from protohaematin compounds. The haematin-*a* (or rather haematins-*a*) obtained from cytochrome-*a* [Negelein, 1933; Roche & Bénévent, 1936] give haemochromogens with an absorption band between 587 and 580 mμ.

When cysteine (B.D.H.), not specially purified, is used instead of metal-free cysteine a different result is obtained. Ferric haemochromogen is reduced, verdohaemochromogen is formed, and, when the amount of cysteine is not too large (3 mg. cysteine-HCl per 6 ml.), the presence of oxyhaematin in the first stage of the reaction is shown by the development of the absorption band at 639 mμ. This band was formed for example when haematin (1·5 mg.) in 20% pyridine (5 ml.) was shaken with a solution of cysteine hydrochloride (3 mg.) in phosphate buffer *p*H 7·6 (0·6 ml.).

The addition of traces of Cu acetate to metal-free cysteine causes rapid formation of verdohaemochromogen; there is also some formation of haemochromogen-*a*. When a solution of haematin (0·4 mg.) in 20% pyridine (0·4 ml.) is shaken with a solution of cysteine hydrochloride (10 mg.), neutralized with Na_2CO_3, in the presence of Cu acetate (0·5 mg.), a considerable amount of verdohaemochromogen is formed during 1 min. shaking with air. Addition of Fe ($FeSO_4$) has the same effect, but its action is much less pronounced than that of Cu; there is a slow formation of oxyhaematin and later of verdohaemochromogen.

In contradistinction to ascorbic acid, cysteine alone is not able to produce oxyhaematin from pyridine-haematin with H_2O_2. To a solution of haematin (0·4 mg.) in 20% pyridine (3 ml.) various amounts of a neutralized solution of metal-free cysteine hydrochloride (1–10 mg.) were added in the bottom part of Thunberg tubes just before the tubes were evacuated, the stoppers of the tubes containing various amounts of H_2O_2 (0·02–0·1 ml. 0·5% solution). In none of the tubes was a band at 639 mμ. formed. When $Na_2S_2O_4$ was introduced through

the side-arms of the tubes, a weak absorption band at 587 mμ. and a very weak band at 612·5 mμ. were observed in addition to the bands of protohaemochromogen.

When however the same experiment was repeated with addition of 0·5 mg. Cu acetate, there was immediate formation of a strong band at 639 mμ. This appears, indeed, to be the best method for preparation of the oxyhaematin. Hence, ascorbic acid is not specific for the formation of this compound any more than for the formation of verdohaemochromogen, and the same reaction mechanism is set in motion by ascorbic acid without metal and by cysteine and Cu and, to a lesser degree, by cysteine and Fe.

With glutathione no formation of the oxyhaematin could be obtained even in the presence of Cu, though Cu distinctly accelerated the formation of haemochromogen *a* by hydrogen peroxide.

Some further observations on the formation and role of hydrogen peroxide in verdohaemochromogen formation

We have studied the effect of catalase under conditions in which we normally obtained a quantitative formation of oxyhaematin. To the mixture of haematin and ascorbic acid was added catalase solution (half the amount which caused very incomplete inhibition of aerobic verdohaemochromogen formation): addition of H_2O_2 now failed to give a trace of the absorption band at 639 mμ. and gas development indicated the destruction of H_2O_2 by the catalase. In the presence of catalase BaO_2 was also inactive.

It is unlikely that concentrations of H_2O_2 greater than those applied in this experiment should be formed when haemochromogen is shaken in air in the presence of ascorbic acid. That this is not so was proved by the following experiment. By the use of "Luminol" (3-aminophthalic acid hydrazide), which gives with H_2O_2 in presence of small amounts of haematin and even in presence of reducing agents a blue chemiluminescence, we detected H_2O_2 in a solution containing (in 8 ml.) 0·4 mg. haematin, 1·6 mg. ascorbic acid, 5 ml. 0·1 *mM*. H_2O_2. No chemiluminescence was observed however when pyridine haemochromogen was shaken with air in the presence of ascorbic acid and luminol, though the formation of verdohaemochromogen proceeded as usual.

This seems to show that hydrogen peroxide formation does not occur in the autoxidation of protohaemochromogen. How then are we to explain the inhibition of the verdohaemochromogen formation by catalase? We have brought evidence which we consider conclusive that the effect of catalase is actually due to the enzyme, and H_2O_2 is its only known substrate. Keilin & Hartree [1936] have recently shown that the H_2O_2 formed when $Na_2S_2O_4$ is shaken with air reacts with certain catalase compounds to form H_2O_2-compounds with them. Here again, luminol does not indicate the formation of H_2O_2 in the solution, though added H_2O_2 acts on luminol in the presence of $Na_2S_2O_4$. The negative result of the luminol test therefore does not exclude the presence of H_2O_2 in a concentration small enough to react with haematin compounds, but it excludes its presence in larger concentrations.

The incomplete inhibition of the aerobic formation of verdohaemochromogen by catalase can thus not be due to the fact that more H_2O_2 is present in these experiments than in the experiment with added H_2O_2 under nitrogen. It is not impossible, though improbable, that there are two simultaneous mechanisms of aerobic verdohaemochromogen formation, one proceeding through H_2O_2 and oxyhaematin and being completely prevented by catalase, and a second mechanism in which H_2O_2 does not participate and which is, therefore, not

affected by catalase. It appears more reasonable to assume that the H_2O_2 is somehow protected against catalase under the conditions of our experiments. The most likely way in which it can be protected is by combination with the haem molecule with which it is to react to form oxyhaematin. If by the autoxidation of haemochromogen not free H_2O_2 is formed, but a haem-H_2O_2 compound, which partially dissociates liberating free H_2O_2, but is rapidly transformed into oxyhaematin, the partial inhibition of this process by catalase can be understood. When free H_2O_2 is added to a mixture of catalase and haemochromogen, it is destroyed rapidly by the former before it can combine with the haemochromogen.

If this hypothesis of the reaction mechanism is correct, we must assume that the haem-hydrogen peroxide compound is formed without free H_2O_2 being an intermediate product. This is in agreement with the second theory, mentioned in the introduction, that a haemochromogen peroxide acts as acceptor of the hydrogen from ascorbic acid but is not in agreement with the radical chain theory of Weiss [1934, 1935].

Observations on the ratio of ferrous to total protohaem iron during the reaction with ascorbic acid

In our preliminary paper [1937, 1] it was stated that the ratio of ferrous to total haemochromogen during the coupled oxidation with ascorbic acid depends on the initial ascorbic acid concentration in an unexpected manner. In some experiments it was found that increased ascorbic acid concentration apparently lowered this ratio. This phenomenon can be explained on the basis of oxyhaematin formation; the ferric form of the oxy-compound does not absorb in the 527 mμ. region, but on reduction with $Na_2S_2O_4$ this band of protohaemochromogen is considerably strengthened. Thus the figure for total haemochromogen appears higher than it actually is, and the experiments show that ascorbic acid accelerates the oxidation of ferrous haemochromogen, not to ferric haemochromogen, but to ferric oxyhaemochromogen.

Discussion

We have shown in this paper that catalase inhibits the formation of verdohaemochromogen from pyridine-protohaemochromogen, though it does not prevent it, whereas the action of free H_2O_2 or haemochromogen (in an atmosphere of N_2) is completely prevented by catalase.

Similar facts have been observed by other authors with regard to the action of H_2O_2 and catalase on the formation of "green pigment" from haemoglobin, and this is, indeed, good evidence in favour of an essential similarity of the "green pigment" formation from haemoglobin and the "green haemin" (verdohaemochromogen) formation from haemochromogen. It has been mentioned earlier that McLeod & Gordon [1922] were able to obtain "green pigment" by the action of H_2O_2 on heated blood agar (in which no catalase is present), whereas in the presence of catalase added H_2O_2 is not active, and catalase does not prevent the green pigment formation with O_2 [Hart & Anderson, 1933]. That catalase, however, inhibits the reaction is evident from the work of Schreus & Carrié [1934]. These authors incubated haemoglobin with liver brei at 37° or higher temperatures (optimum 70°) in the presence of alkaline buffer, and obtained biliverdin from the acetic acid-ether extract. After initial difficulties (the authors did not give experimental details of their method) we have been able to confirm their results. Although the yield is very small, there can be no doubt that the pigment is biliverdin (dehydrobilirubin). The blank was completely

negative. In consideration of the strong reducing power of liver tissue it is extremely unlikely that biliverdin should be formed by secondary oxidation of bilirubin, nor does such a reaction occur during the extraction as the authors assume. It is possible that verdohaemochromogen[1] and not biliverdin is formed under these conditions and is transformed into biliverdin during extraction or treatment with acid. However that may be, verdohaemochromogen is probably an intermediate product. Heat and catalase affect the reaction in the same way as they do verdohaemochromogen formation from pyridine-haemochromogen, catalase inhibiting, but by no means completely, and rise in temperature increasing the rate of oxidation. It is therefore probable that an unstable haem-H_2O_2 compound is formed in this process as in the formation of pyridine-verdohaemochromogen. As an explanation that catalase does not prevent aerobic verdohaemochromogen formation, we have assumed that the haem-H_2O_2 compound is formed directly from a haem peroxide by hydrogen transference from the ascorbic acid:

$$(Fe) + O_2 \rightarrow (Fe)O_2;\ (Fe)O_2 + H_2A \rightarrow (Fe).H_2O_2 + A,$$

where (Fe) means haemochromogen, H_2A ascorbic acid and A dehydroascorbic acid. One might attempt to explain the observed facts by assuming "nascent hydrogen peroxide" to be formed during the aerobic haemochromogen oxidation, as Keilin & Hartree [1935, 2] have done to explain the coupled oxidation of alcohol by dehydrogenase systems. We do not believe that this is a satisfactory explanation. The meaning of the word "nascent" is not clear; nascent hydrogen peroxide may mean activated H_2O_2, or H_2O_2 constantly reformed in small concentration, or H_2O_2 held in combination. The first assumption does not explain the experiments of Keilin (activated H_2O_2 would hardly require the presence of a catalyst for a reaction and it would lose its activation energy in such a combination with the catalyst); it is also unnecessary for the explanation of our experiments in which H_2O_2 does not require activation. The slow delivery effect does not explain the experiments of Keilin, at least if catalase is supposed to be the catalyst for the oxidation of alcohol, since the formation of a catalase-H_2O_2 compound would have to precede this reaction as well as the catalytic H_2O_2 destruction. In our case it might appear to explain the observed facts, since a small amount of H_2O_2 escaping the action of catalase might be supposed to have a noticeable effect when constantly renewed. The catalase inhibition is, however, too small for such an explanation, and other peroxides which slowly liberate hydrogen peroxide and with which Keilin could replace the "nascent hydrogen peroxide" in his experiments, were in our experiment in the presence of catalase as inactive as H_2O_2. Should the third possibility be correct the term "combined" rather than "nascent" hydrogen peroxide would be suitable, and in our case the protection from catalase is best understood by assuming combination with haem, haem being the molecule with which the H_2O_2 is to react, and by assuming the formation of such a compound without intermediate formation of free H_2O_2. The mechanism described above fulfils these conditions and also explains why the reaction occurs only with substances which can be considered as hydrogen donators, but not with other reducers, as $Na_2S_2O_4$.

In pyridine-haemochromogen the six co-ordination positions of the iron atom are filled, four by the porphyrin nitrogens and two by pyridine nitrogens. O_2 must, therefore, remove one pyridine molecule from the Fe atom, as CO does in forming CO-haemochromogen. In an earlier publication it has been

[1] Verdohaematin combines with native globin to a haemochromogen, not to a verdohaemoglobin [Lemberg & Wyndham, 1936; Roche & Bénévent, 1937].

assumed that O_2 enters between the Fe atom and pyridine nitrogen forming a peroxide in which O_2, by the particular way in which it is bound in the compound, becomes a strong oxidizer. Such a formula explains less readily the second step of the oxidation of haematin which we assume in our more recent hypothesis, the hydrogenation of the peroxide to an H_2O_2-compound. To form this substance, one pyridine molecule must be removed completely, since H_2O_2 cannot be inserted between haem iron and pyridine nitrogen. This might therefore occur in the formation of the peroxide. The O_2 must be bound, however, in a different way from that in oxyhaemoglobin.

In contradistinction to oxyhaemoglobin pyridine-haemochromogen peroxide is unstable and breaks down to give ferric haemochromogen in a way which is not yet clear, but which does not require the presence of reducers. When these are present they react with the haem peroxide to give some haemochromogen-H_2O_2 compound in a reaction which proceeds simultaneously with the formation of ferric haemochromogen. There is no evidence that the oxidation of ferrous to ferric haemochromogen is accelerated by the presence of reducers, as we believed at first. The velocity of ascorbic acid oxidation which we can consider as a measure of the rate of oxidation of ferrous to ferric haemochromogen is therefore not decreased by catalase.

Quite different from the autoxidation of pyridine-haemochromogen is the oxidation of haemoglobin to methaemoglobin, since the reaction with O_2 in the absence of reducing substances or systems leads to oxyhaemoglobin, whereas in their presence methaemoglobin arises [cf. Warburg *et al.* 1931, where the earlier literature on the subject is reviewed]. The conditions under which methaemoglobin is formed are, indeed, often the same as those which cause green pigment formation (e.g. in the bacterial systems [Hart & Anderson, 1933]). Bernheim *et al.* [1936] and Bernheim & Michel [1937] have shown that dehydrogenase systems of tissues and other dehydrogenase systems oxidize haemoglobin to methaemoglobin in the presence of oxygen, and that catalase inhibits, but does not prevent the reaction. It is possible that here the H_2O_2 formed by the dehydrogenase systems oxidizes the haemoglobin and being constantly renewed escapes partly the destruction by catalase. It is also possible, however, that the activated substrates transfer their hydrogen to oxyhaemoglobin and thus form an unstable haemoglobin-H_2O_2 compound which breaks down to give methaemoglobin.

The experiments of Heubner *et al.* [1923] on the action of phenylhydroxylamine on oxyhaemoglobin leave no doubt that the two substances react. The catalytic effect of haemoglobin on the oxidation of phenylhydroxylamine and the rapid quantitative reaction of the latter with oxyhaemoglobin in the absence of free O_2 cannot be explained by the action of H_2O_2 formed by autoxidation of phenylhydroxylamine on oxyhaemoglobin, since the autoxidation of phenylhydroxylamine is much slower. The equations

$$HbO_2 + 2C_6H_6.NHOH \rightarrow Hb.H_2O_2 + C_6H_5.N_2O.C_6H_5;$$
$$Hb.H_2O_2 + HbO_2 \rightarrow 2HbOH + O_2$$

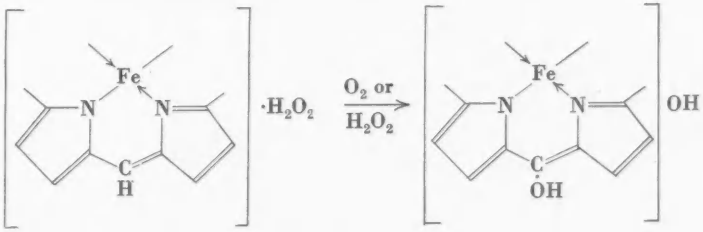
are in agreement with the observations of the authors.

It is also the only explanation applicable to the formation of green pigment from oxyhaemoglobin and ascorbic acid. The possibility had not been excluded by our experiments that the direct autoxidation of pyridine-haemochromogen without participation of H_2O_2 yields verdohaemochromogen when frequently repeated. The formation of green pigment cannot, however, be due to autoxidation of haemoglobin, since even often repeated exhaustion and oxygenation of

haemoglobin does not cause green pigment formation. If this were so, verdohaemochromogen would be formed in the normal course of events in the red cells, and there is no evidence for this. We have to assume that hydrogen donators cause the formation of green pigment by reducing oxyhaemoglobin to an unstable H_2O_2 compound.

More experimental material, however, is required to understand fully the oxidation of haemoglobin to "green pigment" and to methaemoglobin in the presence of reducing substances. For the formation of methaemoglobin the process assumed above is without doubt only one of several (formation by H_2O_2 or by oxidizing substances formed by oxidation of reducing substances).

The third step in the formation of verdohaemochromogen from pyridine-haemochromogen is the formation of the compound with the absorption band at 639mμ. which we consider to be a ferric haematin of an oxyporphyrin, a porphyrin in which oxygen, probably in the form of a hydroxyl group, has been attached to the methene bridge α. We can write:

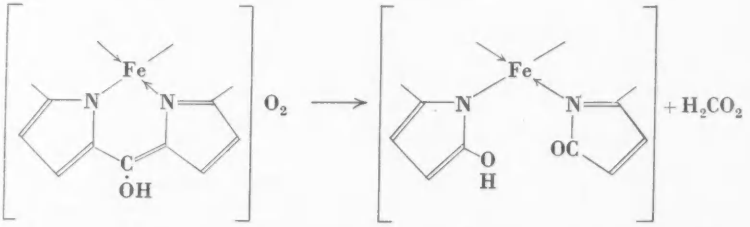


This compound is probably identical with a compound with the same absorption band, observed by Warburg *et al.* [1933] in *Bact. Pasteurianum*, when suspensions of the bacteria were shaken with air in the presence of cyanide. The absorption band at 639mμ. could not be observed under anaerobic conditions with or without cyanide or under aerobic conditions without cyanide. For these reasons Warburg considered the compound to be a cyanide compound of a ferric haematin different from both respiratory ferment and cytochrome. He assumed that it played an important part in the respiration of these bacteria, reducing the ferric form of respiratory ferment and oxidizing the ferrous form of cytochrome-*a*. He believed that in consequence of its high redox potential it should be found in the cell in the reduced form only, even under aerobic conditions, but that cyanide should shift the equilibrium to the ferric side, forming the relatively stable cyanide compound and thus inhibiting respiration. As a weak point of this theory he admitted the fact that no ferric haem-cyanide compound with an absorption band in this region is known.

If, as we believe, this compound is identical with oxyhaematin and is not a cyanide compound, it cannot be a normal constituent of the cells, since its band is not visible in the cells in the absence of cyanide. The action of cyanide in causing its formation must be more complex. Warburg has shown, that the greater the cyanide inhibition of the respiration, the stronger is the absorption band at 639mμ. Catalase, however, is very sensitive to cyanide, and its inactivation may allow the formation of hydrogen peroxide in the cells, which, as we have shown, forms oxyhaematin from protohaematin in the presence of hydrogen donators. This reaction is little inhibited by cyanide, and the absorption spectrum of the bacterial suspension makes the presence of protohaemochromogen probable. The formation of H_2O_2 in *Bact. Pasteurianum* has not been

proved owing to the presence of catalase, but Wieland & Pistor [1936] have made the presence of H_2O_2 and its reaction with alcohol probable in the similar *Acetobacter peroxidans* which is free from catalase, but contains haematin pigments.[1] In the lactic acid bacteria, which are also facultative aerobes but do not contain haematin pigments or catalase, the formation of H_2O_2 has been demonstrated. The respiration remaining in the presence of cyanide may suffice to form the small amounts of H_2O_2 necessary for the oxidation of protohaemochromogen to oxyhaematin. This will be further oxidized, but its formation is much more rapid than its further oxidation. We believe, therefore, that the compound observed by Warburg *et al.* in *Bact. Pasteurianum* is an artificial product arising in the cells only under non-physiological conditions.

The mechanism of the final step, the oxidation of oxyhaematin to verdohaematin, is not yet quite clear. Molecular oxygen is required for this reaction, whereas H_2O_2 either does not react, or destroys completely. The oxygen, already attached to the methene group α of oxyhaematin, makes it more liable to further oxidation (splitting of the porphyrin ring), but free oxyporphyrin is stable and does not break down to give bile pigments. The haem iron is, therefore, necessary for this reaction also. Since, however, the reaction appears to be independent of ascorbic acid concentration and the latter reduces oxyhaematin only slowly, the ferric form appears to be attacked by oxygen without previous reduction, and no satisfactory explanation can be given for the mechanism of such a reaction. Perhaps an oxidation product of ascorbic acid, possessing a stronger reducing power than the acid itself, causes reduction of the oxyhaematin to oxyhaemochromogen; weak absorption bands of oxyhaemochromogen remain, indeed, visible when a solution of it is shaken with air. The reaction would then be a true autoxidation of oxyhaemochromogen, leading partly to oxyhaematin and partly to verdohaemochromogen and several repetitions of this process would be required for a complete transformation of oxyhaematin to verdohaematin. This final step of the reaction we write as follows:



For the action of cysteine in forming oxyhaematin and verdohaematin Cu or Fe is necessary; they are required for the first step of the reaction, the reduction of ferric to ferrous haemochromogen. These metals form complex salts with cysteine which, by a valency exchange of their metal atom with that of haematin iron, perform the reduction of the latter, which cysteine is unable to do alone. The role which the metals play in this reaction deserves consideration. There might be a correlation of this action of the metals, particularly Cu, in blood pigment breakdown and their well-known activity in blood pigment formation. Both Cu and ascorbic acid are necessary to prevent anaemia. What

[1] This peroxidative destruction of alcohol may be similar to the action of "nascent hydrogen peroxide" on alcohol found by Keilin & Hartree.

this correlation may be, however, is still quite obscure, particularly since Cu does not enable glutathione to reduce ferric haemochromogen and to form verdohaemochromogen.

Summary

As intermediate product in the formation of verdohaemochromogen from pyridine haemochromogen by O_2 in the presence of ascorbic acid, a haematin compound with an absorption band at 639mμ. is found, which is characterized as a ferric haematin compound of an oxyporphyrin.

This substance can be formed from protohaemochromogen by the action of hydrogen peroxide, and is probably identical with the compound having the same absorption band, observed by Warburg and co-workers in *Bact. Pasteurianum* under aerobic conditions in the presence of cyanide.

By action of molecular oxygen it is transformed into verdohaemochromogen.

Aerobic verdohaemochromogen formation is inhibited, but not prevented, by catalase, whereas the enzyme completely prevents the formation of the oxyporphyrin-haematin by hydrogen peroxide. The presence of free hydrogen peroxide during the aerobic formation of verdohaemochromogen cannot be proved by other means than the catalase inhibition. From these observations it is concluded that the first steps of the oxidation of protohaemochromogen to verdohaemochromogen are the formation of a haemochromogen peroxide (Warburg) and its hydrogenation (Wieland) to a haem-hydrogen peroxide compound.

The oxidation of ascorbic acid is due to ferric haemochromogen formed by autoxidation of ferrous haemochromogen, and this reaction is independent of the reaction mechanism causing the formation of verdohaemochromogen.

Ascorbic acid in these experiments can be replaced by cysteine + copper or cysteine + iron, not, however, by metal-free cysteine or by glutathione with or without metal. Glutathione and metal-free cysteine cause the formation of a haemochromogen-*a*.

The formation of "green pigment" from haemoglobin and oxygen in the presence of ascorbic acid and other hydrogen donators is discussed and its mechanism is found to be essentially the same. This mechanism is probably responsible for some instances of methaemoglobin formation.

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During the final stages of this research Mr W. H. Lockwood, B.Sc., has collaborated.

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XXV. THE EFFECT OF CO_2 ON THE PRODUCTION OF SUCCINIC ACID BY *BACT. COLI COMMUNE*

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Two theories have been proposed to explain the production of succinic acid during the fermentation of carbohydrates and allied substances by bacteria. The first, suggested by Grey [1924], supposed that in fermentations by *Bact. coli* succinic acid arose by the condensation of two molecules of acetic acid in the presence of a suitable hydrogen acceptor to give one molecule of succinic acid and two atoms of hydrogen. Recently Wood & Werkman [1936] have shown that the propionic acid bacteria form succinic acid from glycerol, and they suggest that it arose from acetic acid.

The second theory was put forward by Virtanen [1925, 1934], and by Virtanen & Karström [1931]. This postulated a cleavage of the hexose molecule into a 4C compound and a 2C compound. The 4C compound was supposed to be changed into succinic acid by an intramolecular rearrangement. Sheffer [1928] constructed carbon balance sheets for the fermentation of glucose by various species of the coli-aerogenes group. He deduced from his results that succinic acid could arise only in the manner suggested by Virtanen.

There have been, as yet, no decisive experiments to test these theories but, during the past year or so, a number of facts have come to light which may have some bearing on the problem of the anaerobic formation of 4C dicarboxylic acids by bacteria. Wood & Werkman [1936] observed that during the fermentation of glycerol by certain species of the propionic acid bacteria there was a fixation of CO_2. They were unable to decide in what form the fixed CO_2 appeared, but suggested that it acted as a hydrogen acceptor in some reaction. They pointed out however that where there was a large utilization of CO_2 a large amount of succinic acid was formed.

In this communication it will be shown that, under strictly anaerobic conditions, the rate of succinic acid formation by washed suspensions of *Bact. coli commune* from sodium pyruvate, glucose and galactose is dependent on the concentration of CO_2 in the medium.

Methods

(1) *Preparation of the suspension.* The strain of *Bact. coli commune* used in this work was provided by the Bacteriological Dept., Sheffield University, through Dr H. A. Krebs. The organism was grown on the surface of meat broth agar, in Roux bottles. After incubation for 14–16 hr. at 38°, the bacteria were washed three times at the centrifuge with distilled water and finally suspended in saline containing $M/20$ bicarbonate [Krebs & Henseleit, 1932], or in $M/20$ phosphate buffer. The dry weight of the organism per ml. was determined by means of a photoelectric turbidimeter [Clifton *et al.* 1935]. All experiments were carried out at 38°.

(2) *The estimation of succinic acid.* A manometric method for the determination of succinc acid involving the use of the succinic dehydrogenase of heart muscle was used. A similar method, based on the same principle, has recently been described by Weil-Malherbe [1937].

The enzyme was prepared afresh each week from pigs' heart by the method of Ogston & Green [1935]. The precipitate obtained from the phosphate buffer extract by adjusting the *p*H to 4·6 gave a homogeneous suspension in 20–25 ml. of phosphate buffer *p*H 7·2. Cresyl blue was the carrier used [see also Weil-Malherbe, 1937]. The wet preparation, like the dry preparation from ox heart used by Weil-Malherbe, oxidized only succinic acid, α-glycerophosphate and α-hydroxyglutaric acid.

The details are due mainly to Dr H. A. Krebs (private communication) and were as follows. The fluid to be examined for succinic acid was brought to *p*H 1–2 with 50% sulphuric acid and extracted for 4 hr. with freshly distilled, dry ether. At the end of the extraction period 2 ml. of *M*/10 phosphate buffer *p*H 7·2 were added to the ethereal extract and the ether distilled off, the succinic acid remaining behind in the buffer. This latter was evaporated down on a water bath to about 0·5 ml. and transferred to a 5 ml. measuring cylinder with *M*/20 phosphate buffer *p*H 7·2. The *p*H was adjusted to about 7·2 with *N*/10 NaOH. The final volume was usually between 2·5 and 3·5 ml. 2 ml. of this solution were added to the main compartment of the manometer cup with 0·1 ml. of a 0·5% solution of cresyl blue. Filter paper-KOH absorbers were used in the centre tube; 0·8 ml. of the enzyme preparation placed in the side bulb was tipped in after equilibration. An enzyme blank was set up with 2 ml. of *M*/20 phosphate buffer *p*H 7·2 instead of the extract. The oxidation was considered complete when the oxygen uptake fell to a value equal to the blank oxygen uptake over a period of 20 min.

Ethereal extraction, although increasing the time required for the estimation, was used for the following reasons: (*a*) A larger amount of succinic acid may be taken for the final estimation owing to the greater concentration, with a consequent gain in accuracy. (*b*) The possible presence of coenzyme would cause substances other than succinic acid (lactic acid, malic acid formed from the fumaric acid) to be oxidized. Coenzyme I is insoluble in ether. (*c*) The estimation of α-glycerophosphoric is prevented since this substance is also insoluble in ether.

Recovery experiments show an accuracy of about 95% (succinic added, 140 μl.; recovered 132 μl., 136 μl., duplicates).

(3) *The estimation of pyruvic acid.* Pyruvic acid was estimated by the carboxylase method of Westerkamp [1933] with the modifications introduced by Krebs & Johnson [1937]. The brewer's yeast was dried at 30° by means of a hot air drier.

(4) *Glucose and galactose* were estimated by the method of Hagedorn and Jensen. For the latter substance the tables published by Gale [1937] were used.

Metabolic quotients

The usual manometric conventions have been adopted for the expression of the rates of metabolic changes, the amounts of metabolites being expressed as μl. (1 m.mol. = 22,400 μl. at N.T.P.). The general formula for the metabolic quotients used is as follows.

$$Q_{\text{metabolite}} = \frac{\mu\text{l. of metabolite}}{\text{mg. dry weight of bacteria} \times \text{hr.}}.$$

Q_s refers to $Q_{\text{succinic acid}}$.

Experimental

(1) *Fermentation in high concentrations of CO_2.* The only substance tested which increased the rate of succinic acid formation from pyruvate was CO_2. Addition of lactate, acetate or formate gave negative results (Table I). In these experiments the total succinic acid formed, and not the rate of succinic acid formation, was measured.

Table I. *Effects of lactate, acetate, formate and CO_2 on the total succinic acid formed from pyruvate*

Warburg manometers, final volume of fluid in the cup 4 ml. The bacteria were suspended in saline containing *M*/20 bicarbonate. 2796 μl. of pyruvate were taken in each experiment and 0·5 ml. *M*/10 solution of the other substrates. Gas phase, unless otherwise stated, N_2/CO_2.

Exp. no.	Substrate	Succinic acid formed μl.
1	Pyruvate	261
	Pyruvate + lactate	274
	Lactate + acetate	0
	Pyruvate + acetate	169
2	Pyruvate	284
	Pyruvate + formate	253
	Pyruvate	263
	Pyruvate (pure CO_2 gas phase)	322

Each Warburg manometer had 3 ml. of the suspension of the organisms in bicarbonate saline. The substrates were tipped in from the side bulb at the end of 20 min. equilibration. The final volume in each cup was 4 ml. The gas phase was either nitrogen containing 5% CO_2 or pure CO_2. Unless otherwise stated N_2/CO_2 gas mixture implies nitrogen containing 5% CO_2. Anaerobiosis was secured by the use of freshly scraped sticks of yellow phosphorus placed in the centre tube of the manometer cup. A blank to which no substrate was added was set up. If the rate of succinic acid formation was being determined, the reaction was stopped at the end of 1 hr. by the addition of 0·5 ml. 50% H_2SO_4; if, however, the total succinic acid formed was being investigated the incubation was continued until the rate of gas evolution in the experimental manometer had fallen to that in the blank. When this occurred the reaction was stopped as described above. The contents of the cups with 0·5 ml. additional H_2SO_4 and 3 ml. of washings were transferred to the extractors for the extraction of the succinic acid.

Raising the concentration of CO_2 in the gas phase increases the total amount of succinic acid formed (Table I). The rate of succinic acid formation is also increased by raising the partial pressure of CO_2 in the gas phase (Table II).

Table II. *Effect of varying the CO_2 concentration on the rate of succinic acid formation from pyruvate*

Volume of fluid 4 ml. Bicarbonate concentration *M*/20. 3366 μl. of pyruvate were used in each experiment and the time was 60 min.

Exp. no.	N_2/CO_2 gas phase		CO_2 gas phase	
	Blank	Experimental	Blank	Experimental
1	Q_{O_2} +1·3	Q_{O_2} +14·0	Q_{O_2} +0·98	Q_{O_2} +21·1
2	Q_{O_2} +1·6	Q_{O_2} +20·7	Q_{O_2} 0·0	Q_{O_2} +29·4
3	Q_{O_2} 0·0	Q_{O_2} +18·9	Q_{O_2} 0·0	Q_{O_2} +31·2

Increasing the concentration of CO_2 in the gas phase has two effects on the fermentation fluid; (*a*) it increases the amount of dissolved CO_2 and (*b*) it lowers the *p*H. At 38° an *M*/20 solution of bicarbonate in equilibrium with nitrogen containing 5% CO_2 has a *p*H of 7·8; whereas the same solution with pure CO_2 has a *p*H of 6·5. Thus the observed increase in the rate of succinic acid formation may be due either to the increase in the amount of dissolved CO_2, or to the fall in *p*H. It is difficult to arrange experiments with the CO_2-bicarbonate buffer to test this point. For a 20-fold increase in the concentration of dissolved CO_2 there is only a 50–60% increase in the rate of succinic acid formation; suggesting that, if CO_2 is utilized by the organism in the synthesis of succinic acid the enzyme or enzymes concerned in its fixation must be almost saturated with their substrate when its concentration in the gas phase is only 5%. Glucose gave no clear-cut increase of succinic acid production when fermented in *M*/20 bicarbonate with a pure CO_2 gas phase.

(2) *Fermentation in low concentrations of* CO_2. If CO_2 is utilized by the organism to form succinic acid from pyruvate it would be expected that a reduction of the concentration of CO_2 would result in a corresponding reduction in the rate of succinic acid formation. In order to test this point the technique employed by Gladstone *et al.* [1935] when investigating the effect of CO_2 on bacterial growth was used. Nitrogen, carefully freed from CO_2 and oxygen, was bubbled through the fluid to remove the CO_2 as formed. Cylinder nitrogen was freed from CO_2 by passage through two wash bottles containing 15% NaOH, and from oxygen by passage over heated, palladized copper wire. The copper was reduced by heating in a current of hydrogen before use. In the first series of experiments the fermentation fluid was contained in a large boiling tube. The gas passed in through a Jena gas distribution tube. By means of a gas outlet the first fermentation tube was connected *via* a wash bottle containing 15% NaOH to a second fermentation tube containing water in place of substrate.

M/20 phosphate buffer *p*H 6·9 boiled just before use to remove dissolved gases was used in these experiments. The final volume in each tube was 40 ml., i.e. 30 ml. of the bacterial suspension and 10 ml. of the substrate or distilled water. The gas was passed through the suspension for 10 min. to equilibrate, the tubes were then momentarily disconnected and the substrate or water added. At the end of a given time the fermentation was stopped by the addition of 5 ml. of 50% H_2SO_4. The total volume of contents and three washings was usually 80–85 ml.; 15 ml. were taken for the estimation of succinic acid.

As a control, similar experiments in which the N_2 was replaced by the N_2/CO_2 gas mixture were carried out.

When N_2/CO_2 is replaced by N_2 the rate of succinic acid formation falls to approximately 40% of that in the N_2/CO_2 (Table III). In other words the removal of CO_2 from the fermentation fluid results in a decrease in the rate of succinic acid formation, as would be expected if CO_2 took part in the formation of succinic acid. Glucose gave similar results to pyruvate (Table III). Here the effect was more marked, the rate in N_2 falling to about 25% of that in N_2/CO_2.

After the above results had been obtained with pyruvate and glucose, Dr H. A. Krebs, in a personal communication, pointed out that galactose gave rise to large amounts of succinic acid when fermented in a bicarbonate-CO_2 buffer. This has been confirmed (Table IV). In addition experiments with galactose show particularly well the reduction in rate of succinic acid formation when CO_2 is rapidly removed from the medium (Table III). Here the rate falls to 0–13% of that in the control in N_2/CO_2.

Table III. *Effect of reducing the concentration of CO_2 in the fermentation fluid on the rate of succinic acid formation*

Time min.	Substrate	N_2 gas phase		N_2/CO_2 gas phase	
		Blank	Experimental	Blank	Experimental
45	Pyruvate, 10 ml. $M/5$	131 μl. $Q_s+2{\cdot}0$	462 μl. $Q_s+7{\cdot}2$	168 μl. $Q_s+2{\cdot}6$	1140 μl. $Q_s+17{\cdot}7$
60	Pyruvate, 10 ml. $M/5$	0 Q_s —	757 μl. $Q_s+6{\cdot}3$	152 μl. $Q_s+1{\cdot}3$	1880 μl. $Q_s+15{\cdot}7$
60	Glucose, 10 ml. $M/10$	0 Q_s —	257 μl. $Q_s+2{\cdot}4$	0 Q_s —	1230 μl. $Q_s+11{\cdot}8$
60	Glucose, 10 ml. $M/10$	85 μl. $Q_s+0{\cdot}9$	504 μl. $Q_s+5{\cdot}3$	102 μl. $Q_s+1{\cdot}1$	1867 μl. $Q_s+19{\cdot}4$
90	Galactose, 10 ml. $M/10$	0 Q_s —	0 Q_s —	0 Q_s —	1180 μl. $Q_s+8{\cdot}2$
60	Galactose, 10 ml. $M/10$	0 Q_s —	176 μl. $Q_s+1{\cdot}9$	158 μl. $Q_s+1{\cdot}7$	1250 μl. $Q_s+13{\cdot}7$

Table IV. *Total succinic acid formed from galactose*

$M/20$ bicarbonate in saline; 4 ml. total vol. in each cup; 1370 μl. of galactose in each experiment.

Blank μl.	Experimental μl.	Experimental − blank μl.	Mol. succinic acid per 100 mol. galactose
164	1115	951	73
127	1070	943	72
120	1228	1108	84·7
70	1193	1123	86

Under these conditions the shift in *p*H when changing from N_2 to N_2/CO_2 is very small. The *p*H of the CO_2-free phosphate ($M/20$) as determined by the quinhydrone method was 6·9; CO_2-free nitrogen was bubbled through during the determination. When N_2 was replaced by N_2/CO_2 the *p*H fell to 6·75. This change is insignificant, more especially as there is a continuous production of free acid during the fermentation.

(3) *The effect of reducing the CO_2 concentration on the rate of substrate utilization.* It might be argued that CO_2 acts by simply increasing the rate of substrate utilization and consequently does not play a direct part in the formation of succinic acid. The observed decrease in the Q_s would then be due to the decreased rate of fermentation in the absence of CO_2.

The rate of substrate utilization under the conditions of the previous set of experiments was therefore determined. This necessitated a modification of the technique which, as it stood, was unsatisfactory when substrate disappearance had to be estimated. The chief objection to the technique was that the final volume of the fluid was so great that the figures for the substrate remaining had to be multiplied by a factor of at least 80, as only 1 ml. samples could be taken; whereas the figures for succinic acid had to be multiplied by a factor of about 5.

An apparatus which would allow working on a much smaller scale was designed. This consisted of a small boiling tube fitted with a side bulb. The gas was passed in through a Jena sintered glass filter stick and escaped through a tube attached to the side bulb. 3 ml. of the suspension containing not more than 20 mg. dry weight of bacteria were used; if thicker suspensions were

employed foaming occurred, and this resulted in a loss of some of the fluid through the outlet tube. 1 ml. of substrate was placed in the side bulb.

The gas was passed through the fluid for 10 min. to equilibrate, the tube disconnected and the substrate tipped into the main compartment. The fermentation was stopped by immersing the tube in a boiling water bath for 5 min. The contents were transferred to a 15 ml. graduated flask and the volume made up to the mark with the washings of the tube. 10 ml. were taken for the succinic acid estimation and the remainder kept for the determination of the amount of substrate unused. In these experiments no controls without added substrate were set up. The results obtained are summarized in Table V.

Table V. *Effect of reducing the CO_2 concentration on the rate of substrate utilization and succinic acid formation*

Time min.	Substrate	N_2 gas phase		N_2/CO_2 gas phase	
		$Q_{substrate}$	Q_s	$Q_{substrate}$	Q_s
60	Pyruvate, 7650 μl.	−266	+12·1	−282	+29·1
60	Pyruvate, 7650 μl.	−288	+ 9·8	−291	+25·6
60	Glucose, 2055 μl.	− 56·1	+ 1·9	− 58·2	+11·4
60	Glucose, 2055 μl.	− 61·2	+ 1·4	− 63·4	+ 8·9
120	Galactose, 992 μl.	− 5·7	0	− 8·1	+ 7·3
120	Galactose, 992 μl.	− 4·8	+ 0·6	− 6·6	+ 7·2

In the cases of glucose and pyruvate there is no significant change in the rate of substrate utilization when compared with the change in rate of succinic acid formation. Galactose on the other hand shows a decrease in the rate of disappearance when CO_2 is removed. This is insignificant when compared with the decrease in rate of succinic acid formation under the same conditions. The discrepancy in the galactose figures may be due to technical difficulties. Galactose was fermented extremely slowly and the amount used was small even though the runs lasted 2 hr. As the amount of galactose disappearing is obtained by difference, small errors are magnified.

The magnitude of the effect appears to vary inversely as the rate of fermentation of the substrate in question. Thus pyruvate is fermented more rapidly than glucose which in its turn is fermented more rapidly than galactose. On the other hand galactose responds more effectively to the removal of CO_2 than does glucose; and glucose shows the effect of removal of CO_2 more than does pyruvate. A possible explanation of this is that when a substrate is fermented rapidly there is a higher concentration of CO_2 in the cell than when a substrate is fermented slowly. And as the rate of removal of CO_2 is the same in both cases there will be a higher concentration of CO_2 in the cells in the presence of the more rapidly fermented substrates, and the effect of its removal will not be so marked.

Discussion and summary

The experiments quoted above, show that (*a*) when a washed suspension of *Bact. coli* ferments pyruvate, the rate of succinic acid formation is increased 50–60% by increasing the concentration of CO_2 in the medium; the total succinic acid formed under the same conditions is also increased; (*b*) that rapid removal of CO_2 from such a suspension fermenting pyruvate, glucose or galactose causes a decrease in the rate of succinic acid formation amounting to 40% of that in the presence of N_2/CO_2 in the case of pyruvate, 25% in the case of glucose and 13% or less with galactose. There is no significant decrease in the rate of substrate utilization when CO_2 is removed from the fermenting fluid.

These facts suggest that possibly CO_2 is involved in the synthesis of succinic acid. No direct evidence for the fixation of CO_2 by this organism has been obtained under the conditions used in these experiments and it would be very unwise to assume such a fixation on the basis of the data presented here. At the moment facts are too few even to permit theorizing on the role of CO_2 in succinic acid formation.

It should be remembered however that Woods [1936] has shown that under certain well-defined conditions *Bact. coli* is able to fix CO_2: These conditions were not fulfilled in this work. A further point of interest arises from the work of Gladstone *et al.* [1935], who showed that *Bact. coli* is unable to grow when CO_2 is rigidly excluded from the medium. The connexion between CO_2 as a growth factor for *Bact. coli* and CO_2 as a possible reactant in the anaerobic breakdown of certain hexoses and allied compounds is at the moment obscure.

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XXVI. THE COUNTERACTION BY FAT OF THE ANTICALCIFYING ACTION OF CEREALS

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MELLANBY [1921; 1925] showed that rickets was produced in young puppies when large amounts of cereals were added to diets relatively poor in calcium and vitamin D. The greater the proportion of cereals in the diet, the greater was the degree of rickets produced. Some cereals appeared to cause worse rickets than others, and these differences bore no relation to the calcium or phosphorus content of the cereals. In all cases, the rachitogenic action could be counteracted by the addition to the diet of more calcium salts or vitamin D. Furthermore the so-called "anticalcifying" or rachitogenic action of the cereals could be destroyed by acid hydrolysis. He therefore postulated the presence of some toxic substance in cereals, which he termd a "toxamin", and which interfered with calcification and could be destroyed by hydrolysis.

Holst [1927] found that he could extract (with 0·5% HCl) from oatmeal a substance which caused rickets in rats when added to a diet of starch which would otherwise give osteoporosis but no rickets. He noted that the ash of oatmeal did not produce rickets when added to such a starch diet, and therefore concluded that the rachitogenic factor was an organic substance destroyed by ashing.

Green & Mellanby [1928] confirmed on rats the previous experiments of the latter on dogs, and Thomas & Steenbock [1936] obtained similar results, although they did not find the same differences in the rachitogenic actions of the various cereals.

Bruce & Callow [1934] suggested that the rachitogenic action of whole cereals might be due to the relatively high proportion (50–80% of the total phosphorus) of phytin-phosphorus which they contain. They found that phytin-P was not well absorbed by the rat, and claimed that the antirachitic potency of various cereals, in a low P diet, was inversely proportional to the percentage of P present as phytin.

They also found that the addition of Na phytate to rachitogenic diets of the type used by Mellanby (i.e. low Ca-high P cereal diets) increased the degree of rickets produced, while a corresponding amount of Na phosphate did not. They concluded that phytin-P has an "anticalcifying" action, and write that "it is possible to assume that this action is the result of lowering of the availability of calcium by binding it as a calcium salt which is at least as insoluble as calcium phosphate".

McCance & Widdowson [1935] found that phytin was not well absorbed from the human intestine, 36–63% being excreted unchanged in the faeces.

Lowe & Steenbock [1936] supported the claim of Bruce & Callow that the phytin-P content of the diet showed an inverse relation to the antirachitic potency, and that phytin was a poor source of P when added to a high Ca-low P diet. They also showed that acid hydrolysis of maize improved its antirachitic properties in proportion to the extent to which its phytin-P was hydrolysed.

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Recently Mottram & Palmer [1937] made experiments in which they counteracted the rachitogenic action of a pure cereal diet by adding sufficient calcium to give a favourable Ca : P ratio. When excess of Ca was added, the diet again produced rickets. They concluded that the rachitogenic action of cereals was not specific, but was due simply to their unbalanced Ca : P ratio. The more extensive researches of Lowe & Steenbock [1936] showed, however, that not only the ratio of Ca to P, but also the percentage of P present as phytin-P in the cereal, determined its rachitogenic potency.

It should, however, be noted that Mellanby [1937] has recently announced that he has made further experiments on the subject and has come to the conclusion that phytin itself cannot account for the rachitogenic (or, as he terms it, "anticalcifying") action of cereals, but that some other organic P compound in cereals probably causes this action.

Furthermore, Bruin & Boumann [1937], unlike Bruce & Callow [1934], found that the giving of sodium phytate instead of sodium phosphate in a high cereal diet (Ca : P = 4 : 1) did not increase the rachitogenic action of the rations, and they concluded that the phytin content is not the cause of the rachitogenic action of cereals. They also found that an aqueous extract of oats promoted rickets when added to a rice diet, the active principle involved being soluble in alcohol.

These experiments are not directly comparable with those of Mellanby and of Bruce & Callow, since the Ca : P ratio was different, and the relatively large amounts of calcium carbonate added to the cereal diets might be expected to create very different conditions in the intestine.

There therefore seems some reason to believe that the rachitogenic action of cereals, in diets which are relatively, although not absolutely, deficient in calcium and vitamin D, is due to the relatively high proportion of phytin (or some other organic P compound) in cereals. This P compound may decrease the availability of the calcium of the diet by forming a calcium salt which is not readily absorbed. The diet would thus be changed by the presence of phytin from one with just sufficient available calcium to allow normal calcification, to a rachitogenic one with too little available calcium.

Recent work on the subject seems to indicate that the interference with calcification is due to a disturbance of the absorption from the intestine of Ca and P, rather than to the presence of a toxic substance. It is therefore necessary to consider what is known concerning the conditions required for intestinal absorption of Ca.

Ca can of course only be absorbed if it is present in the intestine in a water-soluble form. The reaction of the intestinal contents is such that $CaCl_2$ cannot exist, and since there are always phosphates in the intestine, there is a likelihood of insoluble calcium phosphate being formed which is not absorbed. There is, however, a possible way in which a soluble Ca compound can be formed, and that is by combination with fatty acids (which are formed in large quantities during the digestion of neutral fats) to form soluble Ca soaps which are absorbed in conjunction with the bile acids.

The experimental evidence for the influence of fatty acids on calcium absorption was pointed out by Stolte [1912], who spoke of a competition in the intestine between phosphates and carbonates on the one hand and fatty acids on the other for combination with the Ca. When there is too much phosphate present in relation to the fatty acids, insoluble calcium phosphate is formed and the Ca involved is lost to the body.

Klinke [1928] suggested that fatty acids combine in the intestine with Ca to

form Ca soaps which are dissolved by the bile acids to water-soluble and diffusible complexes. He found that by shaking 0·01 *M* deoxycholic acid with $CaCO_3$, 2·6 mg./100 ml. of Ca was dissolved, with $Ca_3(PO_4)_3$ 4·6 mg. %, and with Ca oleate 13·0 mg./100 ml. of Ca was dissolved.

Boyd *et al.* [1932] found that moderate amounts of fat (10%) increase the intestinal absorption of Ca and P, probably by making an acid reaction in the intestine so that Ca soaps are formed, which are well utilized. Hickmans [1924] stated that the absorption of Ca in children was optimal if the food contained a proportion of 0·04–0·08 g. Ca to 1 g. fat.

From the study of the literature cited above, the rachitogenic action of cereal diets appears to be due to interference with the absorption of Ca from the intestine. Now, it was noted that the experimental diets used by Mellanby and others to demonstrate this rachitogenic action of cereals, contained little fat. Since it is known that fats have a great influence on the intestinal absorption of Ca, it seemed probable that the addition of fat to such cereal diets would counteract their rachitogenic tendencies by creating more favourable conditions in the intestine for Ca absorption.

Rat-feeding experiments were therefore made, using the high cereal diets originally described by Green & Mellanby [1928] to produce rickets in young rats. Fat was added to some of the diets and a calorically equivalent amount of starch to others, to test whether the addition of fat alone would prevent the rickets produced by these cereal diets. The quantity of fat added was about 11% of the total weight of food, a proportion which is quite usual in human diets.

Methods

Young albino rats, of the laboratory's own stock, about 4 weeks old, weighing 50–60 g. were used. They were kept in separate glass cages, with metal screen bottoms, and were given water and their appropriate ration *ad lib.* They were weighed weekly, and killed after 33 days when it was seen by roentgenographs that rickets had developed.

The basal diet used, and the proportion of flour to basal diet, were the same as those used by Green & Mellanby [1928] to show the rachitogenic action of cereals in rats. The diet was made up freshly every 4 or 5 days as described by these authors. It consisted of

24 g. extracted casein (Hoffmann-La Roche),
32 g. marmite (yeast extract),
32 g. lemon juice (fresh),
25 g. dried cabbage,
12 g. NaCl.

The dried cabbage was made by shredding fresh cabbages, from which the outer green leaves were removed, and drying it at 55° for 24–36 hr. The dried shreds were then ground to powder and mixed with the other ingredients of the basal diet. Fresh lemons were used for each occasion.

Portions of the freshly made basal diet were then mixed with the various flours plus either starch or fat for the experimental rations, and the cakes so formed were kept in a refrigerator. About 20 g. were given to each animal daily, this quantity being more than any one animal ate per day.

The ratio of basal diet to flour was usually 1 : 3, as in the experiments of Green & Mellanby. Where fat was added, it was added in the proportion of 0·5 part of fat to 1 part of basal diet and 3 parts of flour. The diet therefore

contained 11% fat. In the control diets, fat was replaced by twice the weight of starch (1 part basal, 3 flour, 1 starch) since 1 part of fat was taken to be about equal in energy value to 2 parts of starch.

The results of the feeding tests were estimated by three different methods. Firstly roentgenographs of the hind limbs of anaesthetized animals were made in all cases. Since this technique is so highly developed, it seemed the best method of determining the degree of rickets.

As a second method, the femora were dissected out immediately after death and cut and stained by the line test method as described by Dyer [1931]. The stained bones were then photographed.

As a third method, the ash percentage of the femora was estimated in the first series. The results of this method, however, were less satisfactory than those of the other two. The ash contents are given in Table I as percentage of the fat-extracted dry weight of the bones. The method used to obtain the fat-extracted dry weight of the bones was that described by Chick *et al.* [1926].

The rats were weighed weekly, and the averages of the gains in weight during the experimental period are given in Table I.

Table I

Series	Group	Diet	No. of rats	Gain in wt. g.	End wt. g.	Degree of rickets by		Ash content of femora %
						Roentgenographs	Line test method	
I	I	Basal + brown flour	5	35	91	1(++), 4(+++)	—	40·1
	II	Brown flour + starch	5	26	81	1(0), 4(+++)	—	43·2
	III	Brown flour + lard	5	45	109	5(0)	—	44·4
	IV	White flour	5	23	70	1(±), 1(++), 3(+++)	—	40·5
	V	White flour + starch	5	37	92	5(+++)	—	41·4
	VI	White flour + lard	5	36	89	5(0)	—	44·4
II	I	White flour + starch	5	29	85	2(+), 1(++), 2(+++)	2(+), 1(++), 2(+++)	—
	II	White flour + lard	5	52	108	1(+), 4(0)	1(+), 4(0)	—
	III	White flour + olive oil	5	31	89	1(++), 1(+), 3(0)	2(±), 3(0)	—
III	I	2 parts wholemeal + starch	5	33	93	2(+), 3(++)	1(+), 4(++)	—
	II	2 parts wholemeal + lard	5	45	102	1(+), 1(±), 3(0)	1(+), 3(0), 1 missing	—
IV	I	White bread	5	42	99	1(0), 1(±), 1(+), 1(++), 1(+++)	2(0), 2(++), 1(+++)	—
	II	White bread + lard	5	54	109	5(0)	5(0)	—

Roentgenographs of all animals were made, but since it is not possible to reproduce them all owing to lack of space, the degree of rickets is recorded in Table I.

Experimental procedure. Since the different feeding tests were spread over the period from February to August, they will be considered in separate series. The experimental technique used was the same in all cases, and the feeding period was always 32–34 days.

The series were as follows:

Series I. White and brown flours with (1) starch and (2) lard.

Series II. White flour with (1) starch, (2) lard, and (3) olive oil.

Series III. Wholemeal flour (smaller quantity) with (1) starch, (2) lard.

Series IV. White bread (1) alone, (2) with lard.

The flours used were the following:

(I) "White flour"—wheaten flour of 70–71% extraction from English and Swiss mills.

(II) "Brown flour"—wheaten flour of 85% extraction with 20% rye flour as commonly used in Switzerland.

(III) "Wholemeal"—wheaten flour of 99% extraction from an English mill.

The lard used was good quality pig's lard as sold for human consumption. Lard, olive oil and coconut oil have been shown by Steenbock & Black [1925] to contain no vitamin D.[1]

The results of all the series are given in Table I. The gains in weight and end weights, and in the first series the ash content of the femora, are given as average values of the 5 rats in each group. The degree of rickets as seen by the roentgen and line test methods is given for each rat. The following signs are used to express the degree of rickets: 0 = no rickets, ± = doubtful rickets; + = slight rickets; ++ = medium rickets; and +++ = severe rickets.

The ash content of the femora is given as percentage of the fat-extracted dry weight.

Series I

In the first series 6 groups of 5 rats each were fed with the following rations:

Group I. Brown flour (3 parts) + basal diet (1 part).
Group II. Brown flour (3 parts) + basal diet (1 part) + starch (1 part).
Group III. Brown flour (3 parts) + basal diet (1 part) + lard (0·5 part).
Group IV. White flour (3 parts) + basal diet (1 part).
Group V. White flour (3 parts), basal (1 part) + starch (1 part).
Group VI. White flour (3 parts), basal (1 part) + lard (0·5 part).

On the twenty-fourth day, 2 rats of each group were X-rayed, and it was seen that rickets had developed in the rats with no fat in the diet, i.e. in groups I, II, IV and V. On the thirty-third and thirty-fourth days all the rats were killed (with coal gas) and X-rayed immediately after death.

It will be seen from Figs. 1–12 that the rats of groups I, II, IV and V, whose diets contained brown or white flour with or without starch, all had marked rickets, while those of groups III and VI, whose diets contained lard, had no rickets.

It is not possible to reproduce here the roentgenographs of all the rats in the experiment; but those not shown here were essentially similar in each group to the representative cases reproduced in Figs. 1–12, which were chosen for their approximately equal growth curves.

There were only two exceptions to this uniformity in the degree of rickets in each group, namely rats No. 6 in group II and No. 20 in group IV, both of which showed very mild or no rickets in contrast to the marked rickets of the other rats in these groups. This is probably to be explained by the fact that these two rats gained only 12 and 11 g. respectively during the experimental period of 33 days, while the majority of the rats gained from 20 to 50 g. It is of course well known that rickets is more likely to develop during periods of vigorous growth, and it was the experience of previous authors (e.g. Green & Mellanby) that rats on such a diet only develop rickets when actively growing.

As can be seen from Table I, the rats of groups III and VI, with lard in the diet, grew better on the whole than those of groups I, II, IV and V without fat.

[1] Daily administration of the non-saponifiable fraction of 30 g. of the lard used in these experiments did not cure rickets in rats on a vitamin D-free diet, whilst 0·1 ml. cod liver oil cured them in 10 days.

Fig. 1.

Fig. 2.

Fig. 5.

Fig. 6.

Fig. 3.

Fig. 4.

Fig. 7.

Fig. 8.

Fig. 9.

Fig. 10.

Fig. 11.

Fig. 12.

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This was true for the groups as a whole, as can be seen from Table I. It is therefore clear that the presence of lard afforded a real protection from rickets which was not due to an inhibition of growth.

Table I also shows the ash content of the bones of the various groups of rats. It will be seen that the average values are slightly higher in the two groups which received fat and developed no rickets. While this is in agreement with the roentgenographs, the latter were taken to be a more reliable index of the degree of rickets.

Series II

The second series of experiments was made partly to confirm the results of the first series, and partly to test whether olive oil would have the same action as lard in preventing rickets. Three groups of 5 rats each were therefore fed on the following diets:

Group I. White flour (3 parts), basal diet (1 part), starch (1 part).
Group II. White flour (3 parts), basal diet (1 part), lard (0·5 part).
Group III. White flour (3 parts), basal diet (1 part), olive oil (0·5 part).

After 34 days on these diets, the rats were killed and the hind limbs X-rayed. The tibiae and femora were then dissected out and preserved in formalin for investigation by the line test method.

The results are shown in Table I. It was found that the rats of group I, without fat in the diet, all developed rickets. In group II with lard, 4 rats had no rickets and the remaining one had only very slight rickets. The addition of lard had therefore prevented rickets in 4 out of 5 animals.

Group III, with olive oil, showed no rickets in 3 rats and only slight rickets in the remaining two. The addition of olive oil afforded also, therefore, protection against rickets, although perhaps less complete protection than lard. It may be noted that the addition of lard improved the growth of the animals, as in the first series, while that of olive oil did not.

Series III

A third series of experiments was made in which the proportion of flour in the diet was reduced from 3 parts to 2 parts per 1 part of basal diet. This gave the diet slightly more resemblance to a human diet, although even such a proportion of cereal is much higher than is usually eaten in British diets.

The diets used were as follows:

Group I. Wholemeal (2 parts), basal diet (1 part), starch (1 part).
Group II. Wholemeal (2 parts), basal diet (1 part), lard (0·5 part).

Each group contained 5 rats as usual and all the animals were killed after 32 days. They were then X-rayed, and the femora and tibiae dissected out and preserved in formalin for the line test.

The results are shown in Table I. It will be seen that all the rats in group I, with no fat in the diet, developed rickets, while in group II with lard, 3 rats had no rickets and 2 very slight rickets. As in previous series, the rats receiving lard grew better than those on the fat-free diet; in spite of this more vigorous growth, which would otherwise tend to cause more severe rickets, the protection against rickets was complete in 3 rats and partial in the remaining 2.

Series IV

A fourth series of experiments was made to test whether white bread (which is the form in which cereals are chiefly eaten in British diets) would exert a similar rachitogenic action in these diets, and whether such an action could be prevented by the addition of lard. Fresh white bread, made of wheat flour of about 70% extraction, was bought every 4 or 5 days, and made up into the following diets:

Group I. Bread (4 parts), basal diet (1 part).
Group II. Bread (4 parts), basal diet (1 part), lard (0·5 part).

Five rats were put on each diet, and killed after 32 days. They were then X-rayed, and the tibiae and femora dissected out and preserved in formalin for the line test.

The results are shown in Table I. It will be seen that of the rats in group I without fat, one had bad rickets (+ + +), two had slight rickets (+) and two had none (0). In group II with lard, all five rats were without rickets.

It seems then that white bread has a similar rachitogenic action to white flour in these diets, but that this action is prevented by the presence of lard, as in the case of flour.

A fifth series of experiments was made in which coconut oil was used as the source of fat, but since the rats did not eat this diet well, they grew less well than the control groups on the same diet without fat. The fact that the rats on the coconut oil diet developed less severe rickets than the others is therefore of uncertain significance.

Discussion

In the introduction, experiments of Mellanby and others were quoted showing that a diet which contains only just enough calcium and vitamin D to allow normal calcification, becomes rachitogenic when it includes a high proportion of cereals. The present experiments, using the same diets which Green & Mellanby used for rats, fully confirm this observation.

It seemed probable from the further researches of Bruce and Callow, and Thomas & Steenbock, that the rachitogenic or so-called "anticalcifying" action of cereals in such diets was due to a disturbance of the intestinal absorption of Ca. Bruce & Callow concluded that the high proportion of phytin in cereals caused this interference with Ca absorption by forming an insoluble and therefore non-absorbable Ca compound in the intestine. It is now probable that this substance is not Ca phytate but a Ca phosphate, since Patwardhan [1937] has shown that there is an enzyme in the rat's intestine which splits phytin. Laskowski [1937], in this laboratory, has confirmed this fact and has demonstrated, by direct absorption tests with rats, that the rate of absorption of P from Na-phytate is in proportion to the rate at which the phytate is split by the enzyme into soluble (and therefore absorbable) Na phosphate. In the presence of Ca, however, an insoluble Ca phosphate may be formed which is not absorbed. This is probably the explanation of the observation of Lowe & Steenbock [1936] that phytin is only assimilated in rats when the diet is low in calcium, since a high calcium content would precipitate all the P as insoluble Ca phosphate in the intestine.

To counteract the loss of Ca by precipitation in the intestine, it was necessary to add to the diet some substance which created favourable conditions for the absorption of Ca. Fatty acids are known to exert such an effect by acidifying

the intestinal contents and by combining with Ca to form Ca soaps, which are dissolved by the action of the bile acids and then readily absorbed. Fat was therefore added to the diets to ensure a constant supply of fatty acids in the intestine during the absorption of the food products.

The experimental results justified these expectations, since the addition of 11% fat (free from vitamin D) prevented the rachitogenic action of the cereal diets of Mellanby. Of three fats tested, lard, olive oil and coconut oil, lard gave the best protection against rickets and also the best growth curves; olive oil was rather less favourable to growth and less certain in its prevention of rickets. Coconut oil gave very incomplete protection against rickets, but it also interfered with the appetite and growth of the animals, so that its purely protective action cannot be judged from these experiments.

It seems clear from these results that the rachitogenic action of cereals in such diets is due to the incomplete nature of the diet, namely to deficiency of fat. A normal amount of fat in the diet seems to be important for the solubility in the intestine (and hence for the absorption and utilization) of Ca and P.

This of course does not mean that Ca and P cannot be absorbed in the absence of fatty acids if the conditions in the intestine are favourable. For instance Mellanby and others have shown that normal calcification can be obtained from these cereal diets if the cereal is hydrolysed, if vitamin D is added or if the Ca : P ratio is made optimal by the addition of a Ca salt [cf. Mottram & Palmer, 1937]. In these cases the conditions in the intestine presumably become changed so as to make the absorption of sufficient Ca and P possible.

From a practical point of view, the important fact is that even in a diet containing a high proportion of cereals and relatively little Ca and vitamin D, as in the experimental diets studied, the so-called anticalcifying tendency of the cereals is counteracted by the presence of about 10% fat.

Summary

1. Rickets produced in young rats by a diet containing a high proportion of wheat flour or bread and low Ca and vitamin D contents, is prevented by the addition of 11% lard or olive oil.

2. The explanation is offered that the absorption of Ca from these diets is promoted by the formation of Ca soaps with the fatty acids, while in the absence of fat the Ca combines with the phytin (or its split-product) to form an insoluble compound which cannot be absorbed.

The author wishes to thank Prof. F. Verzár for his advice and criticism throughout this work and for the privilege of working in his laboratory, and Prof. M. Lüdin for the many roentgenographs made in his Department.

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DESCRIPTION OF PLATE III

Figs. 1—12. Roentgenographs of knee-joints of rats.
Figs. 1 and 2. Rats Nos. 3 and 4, of group I (brown flour).
Figs. 3 and 4. Rats Nos. 8 and 9, of group II (brown flour +starch).
Figs. 5 and 6. Rats Nos. 17 and 19, of group IV (white flour).
Figs. 7 and 8. Rats Nos. 23 and 25, of group V (white flour +starch).
Figs. 9 and 10. Rats Nos. 12 and 15, of group III (brown flour +lard).
Figs. 11 and 12. Rats Nos. 26 and 29, of group VI (white flour +lard).

XXVII. THE USE OF SODIUM SULPHATE FOR THE PREPARATION OF CONCENTRATED PROTEIN-FREE TISSUE EXTRACTS

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Sodium sulphate has been used as an alternative to ammonium sulphate for the precipitation of proteins since the last century, and its special advantages were exploited by Pinkus [1901], who drew attention to the importance of the facts that this salt is relatively insoluble at 0°, and that it crystallizes as a decahydrate. But these properties, which Pinkus utilized for the purification of proteins, lend themselves more directly to the isolation of non-protein substances contained in protein solutions or in tissues [M. G. & P. Eggleton, 1932].

Briefly, if 50 g. of anhydrous Na_2SO_4 are added to 100 g. of water at 32°, a saturated solution is formed, and if protein is present it is completely precipitated and may be filtered off. If this saturated solution is now cooled to 0° it separates into 110 g. of $Na_2SO_4.10H_2O$ and 40 g. of 4 % Na_2SO_4 solution. The water-soluble constituents originally present in the 100 g. of water are now contained in 40 g. and free from protein. The principle is applicable without modification to minced muscle tissue, and since 100 g. water are contained in 125 g. muscle, this quantity should yield 40 g. of a protein-free extract, three times as concentrated as in the original tissue.

The following table, taken from a typical experiment shows that this is the case.

Table I

	Concentration in muscle mg. per 100 g.	Concentration in Na_2SO_4 extract mg. per 100 g.	Concentration ratio
Carnosine (by Pauly reaction)	35	112	3·2
Non-protein nitrogen	247	690	2·8
Lactate	250	700	2·8
Total phosphorus	118	303	2·6

The use of trichloroacetic acid, or similar protein precipitant would give about 500 g. of extract, about twelve times more dilute.

We have applied this technique to tissues other than muscle and obtained protein-free extracts from liver, brain, defibrinated blood and milk.

Actually manipulative losses are greater with the Na_2SO_4 technique, but in certain cases this disadvantage is outweighed by the smallness of the bulk to be handled, the rapidity of the process, and the mildness of the reagent employed. 1000 g. finely minced tissue are thoroughly mixed with 400 g. of powdered anhydrous sodium sulphate. The mixture is warmed to 32°, pressed out with the aid of a small fruit press previously warmed to the same temperature and, if necessary, the resulting extract is filtered (under suction). Care must be taken

to prevent much sodium sulphate from crystallizing out in the press or on the filter, as then some of the protein may dissolve and go into the filtrate.

The clear colourless oily solution (about 600 g.) is chilled to 0°, and the mother liquor separated by filtration, or better in a centrifugal crystal dryer. It is an economy to separate first at room temperature, chill the separated fluid to 0°, and filter off the further crop of crystals on a Büchner funnel. Yield 160 g. of extract (50 %).

We obtained the same average yield of 50–60 % of extract from muscle-, liver- and brain-tissues, and defibrinated blood. No doubt this yield can be increased if a more efficient press is available.

One pint of milk (300 g.) mixed with 135 g. anhydrous Na_2SO_4, warmed to 32°, filtered warm on a Büchner funnel, cooled down to 0° and filtered again, yielded 99 g. of extract, corresponding to a yield of over 90 %.

This method of extraction has also been used with success to obtain quickly extracts from blood for pharmacological testing. 100 ml. of blood (37°) is well mixed with 40 g. anhydrous Na_2SO_4 and immediately filtered under suction into a flask cooled in ice. The cooled filtrate is poured away from the Na_2SO_4, $10H_2O$ crystals, and can be injected at once in cases where the presence of 4 % of Na_2SO_4 does not matter. If necessary the extract can be treated with a $CaCl_2$ solution, the $CaSO_4$ filtered off, and the volume made up to six times the original volume of extract. The product is then isotonic and any active principles are present in a concentration about half that in the original blood.

If the yield is important, it is profitable to carry out a second extraction, by taking the tissue residue and crystallized Na_2SO_4, $10H_2O$ left over from the first extraction, adding enough water to make a saturated solution of sodium sulphate at 32°, or preferably the same amount of 4 % Na_2SO_4 solution as the first extract, mixing thoroughly, warming to 32°, pressing and filtering at this temperature, cooling down to 0° and filtering again.

1000 g. muscle on second extraction (750–800 g. tissue residue + 420–440 g. Na_2SO_4, $10H_2O$ + 160 g. 4 % Na_2SO_4 solution) yield again about 160 g. of extract. This second extract is only half as concentrated as the first one, thus corresponding to a yield of 25 % and increasing the total yield from both extractions to 75 %.

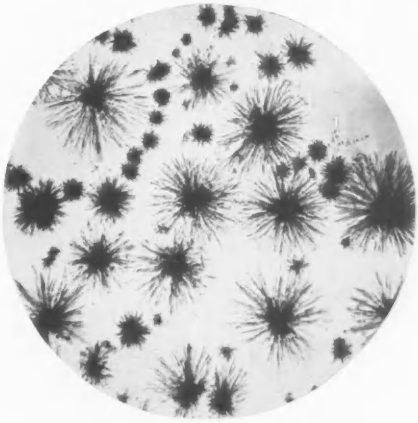
Our original intention in developing this method of extraction was to isolate from fresh muscle several substances, notably creatinephosphoric acid, carnosine and anserine, in order to discover whether they were present in the living tissue in the form of derivatives unstable towards the usual protein precipitants. No evidence of this was obtained in the cases examined.

We have applied the Na_2SO_4 technique also to the isolation of creatine and lactic acid from butcher's meat, and to the isolation of various other substances. Creatine can be precipitated immediately from the extract by addition of acetone, and lactic acid was isolated as the zinc salt by continuous extraction of the acidified extract with ether, removal of the ether and treatment with zinc carbonate [Ghaffar, 1935].

In these cases it is the rapidity of the process and the small bulk to be handled, which present an advantage over other methods of extraction.

Barium creatinephosphate.[1] Twenty Hungarian frogs are kept for a day at 0–4°. They are then pithed, skinned and the hind limbs cut off and minced with Na_2SO_4 and a small addition (totalling 1–2 ml.) of conc. ammonia, to keep the reaction alkaline. More Na_2SO_4 is added at the end, to bring the ratio of Na_2SO_4 to muscle + bone up to 2 : 5.

[1] The experiments on the isolation of this substance were performed in association with Dr S. M. Ling of Peiping.



Barium creatinephosphate × 80.

Barium creatinephosphate under crossed nicols × 60.

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The final extract (60 g. from 375 g. muscle + bone) is treated with an excess of saturated barium acetate solution and the volume is doubled by addition of acetone. After thorough mixing, the precipitate is filtered off on a Büchner funnel or centrifuged, washed successively with 50% acetone and acetone, and finally dried in a desiccator.

The precipitate is extracted thoroughly with water and the extract precipitated with an equal volume of acetone. This is best done by successive extractions with the smallest volume it is convenient to work with. The extraction is rendered less efficient by the adsorption of barium creatinephosphate on the barium sulphate precipitate as was recorded by Fiske & Subbarow [1929], but by a sufficient number of extractions the barium creatinephosphate can be got out from the precipitate. The combined precipitate is filtered off and dried.

The crude barium creatinephosphate is taken up in five times its weight of water, the solution filtered, and methyl alcohol added until a slight but permanent turbidity shows that saturation has been reached. If the side of the vessel is scratched crystallization of the barium salt begins, sometimes immediately, sometimes after a few hours. Recrystallization is effected in the same way.

The yield of once recrystallized salt from a preparation on this scale is about 0·4–0·5 g. By carrying out a second extraction, the yield can be increased to 0·6–0·7 g. For analysis the substance is dried in high vacuum over P_2O_5 at 20°. (Found: C, 13·7; H, 2·8; N, 11·7; P, 8·6; Ba, 37·3%. $C_4H_8O_5N_3PBa$, H_2O requires: C, 13·2; H, 3·0; N, 11·5; P, 8·5; Ba, 37·7%.)

The crystal form is illustrated in Plate IV.

Carnosine and anserine. Both these substances have been isolated from vertebrate skeletal muscle [*v.* Wolff & Wright Wilson, 1935, for further references]. For the isolation of these substances we adopted a simplified technique, working under the mildest possible conditions in order to ascertain whether carnosine and anserine are present in muscle as such or in form of unstable derivatives. As already mentioned, no evidence of the latter could be obtained.

Copper carbonate (freshly prepared from copper sulphate and sodium bicarbonate) is stirred into the Na_2SO_4 extract from muscle, until there is an excess. No heat is necessary. The solution is filtered into a distilling flask and concentrated under reduced pressure (at 30–35°) until the bulk is reduced to one-quarter, or until the copper carnosine (or copper anserine) crystallizes out. It is then set aside overnight, and the crystals filtered off. The yield can be increased slightly by further concentrating the mother liquor or adding acetone.

As an alternative to the concentration under reduced pressure, 2 vols. of acetone may be added to the filtered copper salt solution (or more if necessary) and the copper carnosine (or copper anserine) precipitates as a thick syrup. This on extraction with acetone may crystallize, but usually gives an amorphous solid.

The product from either preparation is dissolved in a minimal amount of conc. ammonia solution, filtered from a colourless crystalline residue, and the ammonia is aspirated off. Crystallization usually begins before all the ammonia is gone, but occasionally it is necessary to leave the ammonia-free solution overnight to crystallize. Recrystallization is effected in the same way.

The copper carnosine and copper anserine were recrystallized three times and dried *in vacuo* over sulphuric acid at 20°.

Horse muscle yielded 0·8–1 g. pure copper carnosine per 1000 g. muscle (extracted twice). (Found: C, 33·6; H, 5·0; N, 16·8%. $C_9H_{14}O_3N_4CuO$, H_2O requires: C, 33·4; H, 4·95; N, 17·3%.)

The pure copper salt was freed from copper by H_2S and the free base converted into the nitrate, following the directions given by Ackermann *et al.* [1929],

for anserine. The nitrate was recrystallized three times from hot dilute alcohol and dried *in vacuo* over P_2O_5 at 100°. M.P. 227° (decomp.), $[\alpha]_D^{20°}$ +23·6° in water (c=3·5, 1 dm. tube). (Found: C, 37·3; H, 5·3; N, 24·0; (N)CH_3, 0·0%. $C_9H_{14}O_3N_4.HNO_3$ requires: C, 37·35; H, 5·2; N, 24·2; (N)CH_3, 0·0%.)

We have also isolated carnosine from the Na_2SO_4 extract of frog muscle. (Found: C, 33·4; H, 4·9; N, 17·0%. $C_9H_{14}O_3N_4CuO$, H_2O requires: C, 33·4; H, 4·95; N, 17·3%.)

Rabbit muscle yielded 1·5–2 g. pure copper anserine per 1000 g. muscle (extracted twice). (Found: C, 37·5; H, 5·1; N, 17·0%. $C_{10}H_{16}O_3N_4CuO$ requires: C, 37·6; H, 5·0; N, 17·5%.)

The pure copper salt was converted into the nitrate in the same way as in the case of carnosine. The nitrate was recrystallized three times from hot dilute alcohol and dried *in vacuo* over P_2O_5 at 100°. M.P. 226° (decomp.), $[\alpha]_D^{20°}$ +14·0° and +12·8° in water (c=3·74 and 6·5, 1 dm. tube). (Found: C, 39·7; H, 5·7; N, 23·4; (N)CH_3, 5·0%. $C_{10}H_{16}O_3N_4.HNO_3$ requires: C, 39·6; H, 5·65; N, 23·1; (N)CH_3, 4·95%.) The carnosine content of the preparation, as measured by the Pauly reaction, was less than 0·5%.

In addition we have isolated anserine from the muscles of sheep and goat. (Found: C, 34·4; H, 5·4; N, 15·4% (sheep) and C, 34·4; H, 5·5; N, 15·4% (goat). $C_{10}H_{16}O_3N_4CuO$, $2H_2O$ requires: C, 33·8; H, 5·6; N, 15·75%.) The carnosine content of these preparations, as measured by the Pauly reaction, was 1·7% (sheep) and 3·7% (goat).

From sheep muscle Smorodinzew [1914] reported the isolation of carnosine. The analyses of minced goat muscle by means of the Pauly reaction indicated a high carnosine content (350 mg. per 100 g., if the Pauly reaction were attributable entirely to carnosine). But with the simplified technique we used one never obtains both carnosine and anserine from the same animal. There is always an appreciable residue of uncrystallizable copper salts, which may be a mixture of anserine, carnosine and other copper salts.

It has been known for some years [Clifford, 1921] that the muscles of invertebrates give no Pauly reaction, and therefore contain no carnosine [*v.* also Flössner, 1932; Broude, 1933]. In order to ascertain whether anserine could be isolated, the isolation technique just described was applied to the flesh of lobster (*Homarus vulgaris*), scallop (*Pecten maximus*), limpet (species not identified) and sea anemone (*Tealia crassicornis*). Only in the case of pecten was a crystalline copper salt obtained. This proved to be copper glycine, and the large yield suggested that there must be about 2% of free glycine in this animal. Both the "quick" and "slow" muscles were used in the preparation, so it is not possible to say if either muscle contains none.

The copper glycine obtained from pecten was recrystallized, and dried *in vacuo* over sulphuric acid at 20°. (Found: N, 12·1%. $(C_2H_5O_2N)_2CuO$ requires: N, 12·2%.)

Glycine was detected by Chittenden [1875] in *Pecten irradians* and later by Kelly [1904] in *P. opercularis*. [For further references see Kutscher & Ackermann, 1936.]

An attempt to isolate carnosine or anserine from the brain of a horse confirmed the negative result of Tschernow, reported by Hefter [1925] in that the extract proved to contain very little of any compound capable of giving a copper salt in neutral solution from $CuCO_3$ and no crystalline substance could be isolated.

SUMMARY

1. A method is described for obtaining concentrated protein-free extracts of animal tissues under very mild conditions, by the use of sodium sulphate.

2. Examples of the application of this technique to the preparation of concentrated extracts from muscle, liver, brain, blood and milk are given.

3. The method has been used to ascertain whether creatinephosphate, carnosine and anserine are present in muscle in free condition or in unstable combination with other tissue constituents. No evidence of such unstable combinations was obtained.

4. Barium creatinephosphate obtained by this method from fresh muscle crystallizes as soon as it is separated from insoluble barium salts. The yield is sufficiently good for the method to be recommended for the isolation of small quantities of pure creatinephosphate.

5. In confirmation of earlier workers carnosine has been isolated from the muscles of the horse and anserine from the muscles of the rabbit. Carnosine has also been isolated from frog muscle, and anserine from the muscles of sheep and goat.

6. Certain other applications of the technique are described.

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